CONCLUSION GENERALE

5. CONCLUSION GENERALE

Le but de ce travail était d'évaluer la place de la PCR *Aspergillus* dans le diagnostic de l'aspergillose invasive.

La première étude réalisée sur les lavages broncho-alvéolaires a établi clairement la nécessité d'inclure la PCR *Aspergillus* dans le diagnostic de l'aspergillose invasive. Pour que ce test soit utile, il faut cependant que la réponse soit rendue dans les 24 à 48 heures après la réception du prélèvement. Or, à l'heure actuelle, seules les nouvelles technologies permettant de détecter les produits d'amplification en temps réel offrent cette possibilité. En effet, ces techniques plus rapides permettent d'augmenter la fréquence des analyses et par conséquent la diversité des recherches par PCR.

Dans le cadre de pathologies opportunistes où la rapidité du diagnostic étiologique est souvent cruciale, l'association de la recherche de cibles différentes est un atout appréciable. On peut ainsi envisager de rechercher par PCR, en association, *Aspergillus*, Cytomégalovirus, Toxoplasme, *Pneumocystis carinii, le virus herpes simplex...* sur le même prélèvement. Le développement de puces à ADN applicables au secteur de la microbiologie (ce qui est loin d'être encore le cas) rendra la batterie infectieuse investiguée encore plus large.

Cependant, si la recherche au niveau des lavages broncho-alvéolaires est importante dans le cadre de l'apparition de troubles respiratoires chez un patient immunodéprimé, la détection de l'agent infectieux, avant l'apparition de signes cliniques notables permettra de traiter les pathologies infectieuses de façon plus efficace. Dans le cadre des aspergilloses invasives, la détection de l'antigène galactomannane est pour le moment le seul paramètre qui semble toutefois utile selon les données de la littérature. La PCR *Aspergillus* ne fait pas l'unanimité des publications. L'étude réalisée ici sur modèle animal a permis de montrer que la PCR *Aspergillus* était outil complémentaire, à réaliser en parallèle avec l'antigénémie galactomannane, dans la mesure où les deux tests ne sont pas toujours positifs en même temps.

En effet, peu d'études portent sur le suivi de patients immunodéprimés par PCR *Aspergillus* avec détection simultanée de l'antigène galactomannane. Peu de centres, en effet, réalisent la recherche d'ADN *Aspergilllus* par PCR, arguant le risque important de contamination des réactifs utilisés en PCR et donc de faux positifs. La commercialisation de kits PCR complets permettra sans doute de

remédier à ce problème. Cependant, pour la plupart des microbiologistes, la priorité de la détection des champignons in vasifs vient après celle de la recherche des bactéries et des virus.

Les perspectives immédiates de ce travail sont donc la développement d'une PCR en temps réel et sa comparaison aux deux techniques précédemment développées. Son évaluation sur tous les liquides biologiques sera nécessaire à la fois en terme de diagnostic ponctuel que dans le cadre de suivi de patients à risque en parallèle avec d'autres recherches de pathogènes opportunistes.

D'autre part, une meilleure connaissance du génome d'*Aspergillus* qui fait l'objet d'un séquençage dans le cadre d'un projet européen, permettra sans doute d'améliorer la définition des cibles à utiliser lors du choix des amorces et donc de rendre les techniques PCR encore plus performantes.

Ainsi, le diagnostic et le traitement de la pathologie aspergillaire sont en pleine évolution. D'une part l'arsenal thérapeutique s'ouvre à de nouvelles molécules moins toxiques que le « gold standard » l'amphotéricine B et néanmoins tout aussi efficaces sinon davantage. D'autre part les outils moléculaires qui doivent désormais faire partie de la routine des laboratoire hospitaliers ouvre la perspective d'un diagnostic plus rapide ce qui augmente les chances de succès d'une chimiothérapie plus précoce et mieux ciblée.

BIBLIOGRAPHIE

6. BIBLIOGRAPHIE

1. Abruzzo G.K., C. Gill, A. Flattery, L. Kong, C. Leighton, J. Smith, V. Pikounis, K. Bartizal, H. Rosen. 2000. Afficacy of the echinocandin caspofungin against disseminated aspergillosis and candidiasis in cyclophosphamide-induces immunosupopressed mice. Antimicrob. Agents Chemother., 44:2310-2318.

2. Aisner J., Schimpff S.C., Bennett J.E., Young, P.H Wienik P.H. 1976. *Aspergillus* infections in cancer patients. Association with fire proofing materials in a new hospital. Jama, 235:411-412.

3. Aisner J., J. Murillo, S.C. Schimpff and A.C. Steere. 1979. Invasive aspergillosis in acute leukaemia: correlation with nose cultures and antibiotic use. Ann. Intern. Med., 90:4-9.

4. Aminati R., A. Sato et al., .1992. Effects of *Aspergillus* species cultures filtrates on human respiratory ciliated epithelium *in vitro.* Am. Rev. Resp. Dis., 145:45-48.

5. Aminati R., G. Taylor, E. N. Elezis, C. Llewellyn-Jones, J. Mitchell, F. Kuze, P.J. Cole,, and R. Wilson. 1995. Purification and characterisation of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect. Immun. 63:3266-3271.

6. Annaissie E.J., S.F. Costa. 2001. Nosocomial aspergillosis is waterborne. Clin. Infect. Dis., 33:1546-1548.

7. Ansorg R., E. Heinstschel von Heinegg, P.M. Ratt. 1994. *Aspergillus* antigenuria compared to antigenemia in bone marrow transplant recipients. Eur. J. Clin. Microbiol. Infect. Dis., 13:582-589.

8. Arikan S., M. Lozano-Chiu, V. paetznick, S. nangia, J.H. Rex. 1999. Microdilution susceptibility testing of amphotericin B, itraconazole, and voriconazole against clinical isolates of *Aspergillus* and fusarium species. J. Clin. Microbiol., 37:3946-3951.

9. Arrese Estrada J., Stynen D., Goris A. and G.E. Pierard. Identification immuno-histochimique d'*Aspergillus* par l'anticorps EB-A1. Annal. Pathol., 10 :198-200.

10. Asciuglu S., J.H. Rex, B. De Pauw, J.E. Bennett, J. Bille, F. Crockert, D.W. Denning, J.P. Donnelly, J.E. Edwards, Z. Erjavec, D. Fiere, O. Lortholary, J. maertens, J.F. Meis, T.F. Patterson, J. Ritter, D. Selleslag, P.M. Shah, D.A. Stevens and T.J. Walsh, on behalf of the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer

and Mycoses Study Group of the National Institute of Allergy and infectious diseases. 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus.Clin. Infect. Dis., 34:7-14.

11. Badillet G., C. de Bièvre, E. Guého. Champignons contaminants des cultures. Champignons opportunistes. Atlas clinique et biologique. Tome II : champignons filamenteux. Varia (Eds), Paris, 1987.

12. Bardana E.J. 1980. The clinical spectrum of aspergillosis . 2. Epidemiology, pathogenicity, infections in animals and immunology of *Aspergillus*. Crit. Rev. Clin. Lab. Sci, 13-21:84.

13. Bart-Delabesse E., A. Marmorat-Khuong, J.M. Costa, M.L. Dubreuil-Lemaire, S. Bretagne. 1996. Detection of *Aspergillus* DNA in bronchoalveolar lavage fluid of AIDS patients by polymerase chain reaction. Eurt. J. Clin. Microbiol. Infect. Dis., 15, 24.

14. Beaune G., F. Bienvenu, Pondarre C., Bienvenu J., Souillet G. 1998. Serum procalcitonine riese in only slight in two cases of disseminated aspergillosis. Infection 26:168-169.

15. Becker M.J., S. De Marie, D. Willemse, H.A. Verbrugh, I. Bakker-Woudenberg. 2000. Quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis in an experimental rat model. J. Clin. Microbiol., 38:1434-1438.

16. Berenguer J.E., M.C. Allende, J. Lee, K. Garret, C. Lyman, N.M. Ali, J. Bacher, P.A. Pizzo and T.J. Walsh. 1995. Pathogenesis of pulmonary aspergillosis-granulocytopenia versus cyclosporine-methylprednisolone induced immunosuppression. Am. J. Respir. Crit. Care Med., 152:1079-1086.

17. Bocquet P., G. Brücker. 1994. Lutte intégrée contre l'aspergillose au niveau d'un hôpital ou d'un groupement hospitalier. Pathol. Biol., 42, 730-731.

18. Bodey G.P., Vartarian S. Aspergillosis. 1989. Eur. J. Clin. Microbiol. Infect. Dis., 8, 413-437.

19. Bodey G.P., B. Bueltmann, W. Duguid, D. Gibbs, H. Hanak, M. Hotchi, G. Mall, P. Martino, F. Meunier, S. Milliken, S. Naoes, M. okudaira, D. Scevola, J. Van't Wout. 1992. Fungal infections in cancer patients: an international autopsy survey. Eur. J. Clin. Microbiol. Infect. Dis., 11:99-109.

20. Boggaerts M., D.J. Winston, E.J. Bow, G.Garber, A.C. Rebolli, A.P. Schwarer, N. Nowitzky, A. Boehme, E. Chwetzoff, K. De Beule, Itraconazole study group. 2001. Intraveinous

and oral itraconazole versus intraveinous amphotericin B deoxyclolate as empiric therapy for persisitent fever in neutropenic patients with cancer who are receiving broad-szpectrum antibacterial therapy. 2001. Ann. Int. Med., 135:412-422.

21. Burnie J.P. and R.C. Matthews. 1991. Heat shock protein 88 and *Aspergillus* infection. J. Clin. Microbiol., 25:5-9.

22. Bretagne, S., J.M. Costa, A. Marmorat-Khuong, F. Poron, C. Cordonnier, M. Vidaud, and J. Fleury-Feith. 1995. Detection of *Aspergillus* species DNA in bronchoalveolar lavage samples by competitive PCR. J. Clin. Microbiol. 33:1164-1168.

23. Bretagne, S., A. Marmorat-Khuong, , J. Kuentz, J.P. latgé, E. bart-Labesse, and C. Cordonnier. 1997. Serum galactomannan antigen testing by sandwich ELISA: practical use in neutropenic patients. J. Infect., 35:7-13.

24. Bretagne, S., J.M. Costa, E. Bart-Delabesse, N. Dhédin, C. Rieux, and C. Cordonnier. 1998. Comparison of serum galactomannan antigen detection and competitive polymerase chain reaction for diagnosing invasive aspergillosis. Clin. Infect. Dis. 26: 104-112

25. Cacciaoupuoti A., D. loebenberg, E. Corcoran, F. Menzel, E. Moss, C. Norris, M. Michalski, K. Raynor, J. Halpern, C. mendrick, B. arnold, B. Antonacci, R. Parmegiani, T. Yarosh-Tomaine, G. Miller, R. Hare. 2000. *In vitro* and *in vivo* activities of SCH 56592 (posaconazole), a new triazole antifungal agent, against *Aspergillus* and *Candida*. Antimicrob. Agents Chemother., 44:2017-2022.

26. Caillot D., O. Casanovas, A. Bernard, J.F. Couaillier, C. Durand, B. Cuisenier, E. Solary, F. Piard, T. Petrella, A. Bonnin, G. Couillault, M. Dumas and H. Guy. 1997. Improved management of invasive pulmonary aspergillosis in neutropenic patients using early thoracic computed tomographic scan and surgery. J. Clin. Oncol. 15:139-147.

27. Caillot D., H. Bassaris, A. McGeer, C. Arthur, H.G. Prentice, W. Seifert, and K. De Beule. 2001. Intraveinous itraconazole followed by oral itraconazole in the treatment of invasive pulmonary aspergillosis in patients with haematologic malignancies, chronic granulomatous disease, or AIDS. Clin. Infect. Dis., 33:e83-e89.

28. Chandler F., W. Kaplan, L. Ajello. A color atlas and text book of the histopathology of mycotic diseases.Wolfe medical publication, Ltd, 1980.

29. Chuchana P. 1991. Réaction de polymérisation en chaîne : méthodologie générale. Ann. Biol. Clin., 50:703-708.

30. Cohen J. 1991. Clinical manifestations and management of aspergillosis in the immunocompromised patient, Fungal infection in the compromised patient, eds Warnock D.W., Richardson M.D., 2nd edn, John Wiley and Sons, Chichester, 117-152.

31. Collective name: Center for disease control and prevention; the infectious diseases society of America; the American society of blood and marrow transplantation. 2000. Guidelines for preventing opportunistic infections among hematopoietic stem cell transplant patients. Biol. Blood Marrow Transplant, 6:659-713; 715;717-727; quiz 729-33.

32. Commers J., K.J. Rochibaud, P.A. Pizo et al.1984. New pulmonary infiltrates in granulopenic patients being treated with antibiotics. Pediatric infectious diseases 3:423-428.

33. Costa C., D. Vidaud, M. Olivi, E. Bart-Delabesse, m. Vidaud and S. Bretagne. 2001. Development of two real-time quantitative TaqMan PCR assays to detect circulating *Aspergillus fumigatus* DNA in serum. J. Microbiol. Methods, 44:263-269.

34. Dannaoui E., F. Persat, M.F. Monier., E. Borel, M.A. Piens, S. Picot. 1999. Use of spectrophotométric reading for *in vitro* antifungal susceptibility testing of *Aspergillus* spp. Can J. Microbiol., 45:871-874.

35. Debeaupuis J.P., Sarfati J., Chazalet V., Latgé J.P. 1997. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infect. Immun., 19:3080-5.

36. De Bièvre C. *Aspergillus*. Cours de Mycologie médicale 1989. Institut Pasteur de Paris.

37. Deeg H.J., R. Strob, E.D. Thomas, et al. 1985. Cyclosporine as prophylaxis for graft-versus-host disease: a randomized study in patients undergoing marrow transplantation for acute nonlymphoblastic leukaemia. Blood, 65:1325-1334.

38. De Hoog G.S. and J. Guarro. 1995. Atlas of clinical fungi, Centraalbureau voor Schimmelcultures, Baarn, 720.

39. Denning D.W. D.A. Stevens. 1990. Antifungal and surgical treatment of invasive aspergillosis: revue of 2.121 published cases. Rev. Infect. Dis., 12 :1147-1201.

40. Denning D.W., K. Venkateswarlu, K.L. Oakley,, M.J. Anderson, N.J. Manning, D.A. Stevens, D.W. Warnock, and S.L. Kelly. 1997. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother., 41 :1364-1368.

41. Denning D.W. , E.G. Evans, C.C. Kibbler, M.D. Richardson, M.M. Roberts, T.R. Rogers, D.W. Warnock, R.E. Warren. 1997. Guidelines for the investigation of invasive fungal infections in haematological malignancy and solid organ transplantation. Eur. J. Clin. Microbiol. Infect. Dis., 16 :424-436.

42. Denning D.W. 1998. Aspergillosis. Clin. Infect. Dis., 26 :781-803.

43. De Repentigny L., S. Petitbois, M. Boushira, E. Michaliszyn, S. senechal, N. Gendron and S. Montplaisir. 1993. Acquired immunity in experimental murine aspergillosis is mediated by macrophages. Infect. Immun., 61:3791-3802.

44. De Repentigny L. 1992. Serodiagnosis of candidiasis, aspergillosis and cryptococcosis. Clin. Infect. Dis., 14(suppl): S11-S22.

45. Einsele, H., H. Hebart, G. Roller, J. Löffler, I. Rothenhofer, C.A. Muller, R.A. Bowden, J.A- Van Burik, D. Engelhard, L. Kanz, and U. Schumacher. 1997. Detection and identification of fungal pathogens in blood by using molecular probes. J. Clin. Microbiol. 35: 1353-1360.

46. Escott G.M., D.J. Adams. 1995. Chitinase activity in human serum and leukocytes. Infect. Immun., 63:4770-4773.

47. Espinell-Ingroff A. 1998. Comparison of in vitriol activities of the new triazole SCH56592 and echinocandins MK-0991 (L-743, 872) and LY303366 against apportunistic filamentous and dimorphic fungi and common and emerging yeast pathogens. J. Clin. Microbiol., 36:1950-2956.

48. Espinell-Ingroff A., M. Bartlett, V. Chaturvedi, M. Ghannoum, K.C. Hazen, M.A. Pfaller, M. Rinaldi, T.J. Walsh. 2001. Optimal susceptibility testing conditions for detection of azole resistance in *Aspergillus* spp.: NCCLS collaborative evaluation. Antimicrob. Agents Chemother., 45:1828-1835.

49. Fontaine T., C. Simenel, G. Dubreucq, O. Adam, M. Delepierre, J. Lemoine, C. E. Vorgias, M. Diaquin, J.P. Latgé. 2000. Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. The J. Biol. Chem., 275:27594-27607.

50. Frosco M. T. Chase and J.D. McMilan. 1992. Purification and properties of the elastase from *Aspergillus fumigatus*. Infect. Immun., 60:728-734.

51. Furman A.C., K.A. Sepkowitz and R. Soave. Acquired immunodeficiency syndrome; p.716-784. *in* R. Reese and R.F. Betts (ed), Practical approach to infectious diseases, 4th ed., Little Brown and company, Boston, Massachusetts.

52. Gage A., Dean D.C. 1970. Aspergillus infection after cardiac surgery. Arch. Surg., 101-384-387.

53. Gefter W., S. Albelda S., G. Talbot, S. Gerson, P. Cassileth, W. Miller. 1984. Invasive pulmonary aspergillosis and acute leukaemia. Limitations in the diagnostic utility of the air crescen,t sign. Radiology, 157:605-610.

54. Golbang N., J.P. Burnie and R.C. Matthews. 1999. A polymerase chain nreaction enzyme immunoassay for diagnosing infection caused by *Aspergillus fumigatus*. J. Clin. Pathol., 52:419-423.

55. Goodley J.M., Y.M. Clayton, J.R. Hay. 1994. Environnemental sampling for aspergilli during building construction on a hospital site. J. Hosp.Infect., 26:27-35.

56. Grillot R., B. Lebeau, P. Ambroise-Thomas. 1994. L'aspergillose invasive: conduite du diagnostic mycologique. Patholo. Biol., 42 :675-682.

57. Grocott R.G. 1955. A stain for fungi in tissue sections and smearsusing Gomori's methenamine-silver nitrate technique. Am. J. Clin. Pathol., 25:975-979.

58. Guillemain R., V. Lavarde, C. Amrein, P. chevalier, A. Guinvarc'h and D. Glotz. 1995. Invasive aspergillosis after transplantation. Transplant. Proc., 27:1307-1309.

59. Hagensee M.E., J.E. Bauwens, B. Kjos, R.A. Bowden. 1994. Brain abcess followingmarrow transplantation: experience at the Fred Hutchinson cancer research Center, 1984-1992. Clin. Infect. Dis., 19:402-408.

60. Hawksworth D.L. Kingdom fungi: fungal phylogeny and systematics, P. 43-65. *In* Microbiology and microbial infections. Volume 4. Medical mycology. Topley and Wilson's. L. Ajello & R.J. Hay (eds), 9th ed., London, 1998.

61. Haynes K.A., and T.R. Rogers. 1994. Retrospective evaluation of a latex agglutination test for diagnosis of invasive aspergillosis in immunocompromised patients. Eur. J. Clin. Microbiol. Infect. Dis., 13 :670-674.

62. Hayette M.P., D. Vaira, F. Suzin, P. Boland, G. Christiaens, P. Melin and P. de Mol. 2001. detection of aspergillus species DNA by PCR in bronchoalveolar lavage fluid. J. Clin. Microbiol., 39:2338-2340.

63. Hearn V.M.1988. Serodiagnosis of Aspergillosis., Pp.43-71. *In* H. Vanden Bossche, D.W.R. Mackenzie and G. Cauwenbergh (ed.), *Aspergillus* and aspergillosis. Plenum Press, New York, N.Y.

64. Hennequin C., N. Benailly, C. Silly, M. Sorin, P. Scheinmann, G. Lenoir, J.L. Gaillard, P. Berche. 1997. *In vitro* susceptibilities to amphotericin B, itraconazole and miconazole of filamentous fungi isolated from patients with cystic fibrosis. Antimicrob. Agents Chemother., 41:2064-2066.

65. Hensel M., H.N. Arst, Aufauvre-Breown A. and D.W. Holden. 1998. The role of the *Aspergillus* fumigatuss area gene in invasive pulmonary aspergillosis. Mol. Gen. Genet., 258:553-557.

66. Herold C.J., J. Kramer, K. Sertl, P. Kahls, R. Maller, H. Imhof, D. Tscholakoff. 1989. Invasive pulmonary aspergillosis: evaluation with MR imaging. Radiology, 173:717-721.

67. Hruban R.H., M.A. Meziane, E.A. Zerhouni, P.S. Wheepler, Dumler J.S., G.M.. Hutchings. 1987. Radiologic-pathologic correlation of the halo sign in invasive pulmonary aspergillosis. J. Comput. Assist. Tomogr. 11:534-536.

68. Jaffe H.W., D.J. Bregman, R.M. Selik. 1983. Acquired immunedeficiency syndrome in the United States: Th efirst 1000 cases. J. Infect. Dis., 148:339-345.

69. Johnson E., K. Oakley, S. Radford, C. moore, P; warn, D. Warnock, D. Denning. 2000. Lack of correlation of *in vitro* amphotericin B susceptibility testing with outcome in a murine model of *Aspergillus* infecion. J. Antimicrob. Chemother., 45:85-93.

70. Jones, M.E., A.J. Barnes, B.A. Oppenheim, P. Balagopal, G.R. Morgenstern., and J.H. Scarffe. 1998. PCR-ELISA for the early diagnosis of invasive pulmonary *Aspergillus* infection in neutropenic patients. J. Clin. Pathol. 51, 652-656.

71. Kahn F., Jones M.J., England D.M. 1986. The role of broncho-alveolar lavage in the diagnosis of invasive pulmonary aspergillosis. Am;. J. Clin. Pathol., 86:518-523.

72. Kami M., T. Fukui, S. Ogawa, Y. Kazuyama, U. Machida, Y. Tanaka, Y. Kanda, T. Kashima, Y. Kamazaki, T. Hamaki, S. Mori, H. Akiyama, Y. Mutou, H. Sakamaki, K. Osumi, S. Kimura, H. Hirai. 2001. Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. Clin. Infect. Dis., 33:1504-1512.

73. Kappe R., A. Schulze-Berge A.n H.G. Sonntag. 1996. Evaluation of eight antibody tests and one antigen test for the diagnosis of invasive aspergillosis. Mycosis, 39:13-23.

74. Kappe R., D. Rimek. Laboratory diagnosis of *Aspergillus fumigatus*-associated diseases, p. 88-104. Dans "Contribution to microbiology, Vol.2, *Aspergillus fumigatus* Biology, clinical aspects and molecular approaches to pathogenicity". Brackage A., B. Jahn, A. Schmidt. (Ed.), 1999, Karger, Basel, Switzerland.

75. Kawamura S., S. Maesaki, T. Noda, Y. Hirakata, K. Tomono, T. Tashiro, S. kohno. 1999. Comparison between PCR and detection of antigen in sera for diagnosis of pulmonary aspergillosis. J. Clin. Microbiol., 37:218-220

76. Koshland D.E. 1989. The molecule of the year. Science, 246:1541.

77. Kozel T.R. 1996. Activation of the complement system by pathogenic fungi. Clin. Microbiol. Rev., 9:34-46.

78. Kramer M.R., D.W. Denning, S.E. Marshall, Ross D.J., G. Berry, N.J. Lewinston, D.A. Stevens, and J. Theodore. 1991. Ulcerative tracheobronchitis after lung transplantation. A new form of invasive aspergillosis. Am. Rev. respir. Dis., 144:552-556.

79. Kuhlman J.E., E.K. Fishman, S. Siegelman. 1985. Invasive pulmonary aspergillosis in acute leukaemia: characteristic findings on CT, the CT halo sign, and the role of CT in early diagnosis. Radiology, 157:611-614.

80. Kwok S. and R. Higuchi. 1989. Avoiding false positives with PCR. Nature, 339 :237-238.

81. Lajonchère J.P., M. Feuillade De Chauvin. 1994. Contamination aspergillaire : évaluation des mesures de prévention et surveillance de l'environnement. Pathologie biologie, 42 :718-729.

82. Lass-Flörl, J. Aigner, E. Gunsilius, A. Petzer, D. Nachbaur, G. Gastl, H. Einsele, J. Löffler, M. Sdierich and R. Würzner. 2001. Screening for *Aspergillus spp.* using the polyumerase chain reaction of whole blood samplesfrom patients with haematological malignancies. Br. J. Haematol, 113:180-184.

83. Latgé J.P. 1995. Tools and trends in the detection of *Aspergillus fumigatus*. Curr. Top. Med. Mycol., 6:245-281.

84. Latgé J.P. 1999. *Aspergillus* and aspergillosis. Clin. Microbiol. Rev., 12:310-350.

85. Levitz S.M., T.P. Farrell. 1990.Human neutrophil degranulation stimulated by *Aspergillus fumigatus*. J. Leukoc. Biol., 47:170-175.

86. Loeffler, J., H. Hebart, U. Schumacher, H. Reitze, and H. Einsele. 1997. Comparison of different methods for extraction of DNA of fungal pathogens from cultures and blood. J. Clin. Microbiol. 35: 3311-3312.

87. Loeffler J., H. Hebart, S. Sepe, U. Schumacher, T. Klingebiel, and H. Einsele. 1998. Detection of PCR-amplified fungal DNA by using a PCR-ELISA system. Medical mycology, 36:275-279.

88. Loeffler J., N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele. 2000. Quantification of fungal DNA by using fluorescence energy transfer and the light cycler system. J. Clin. Microbiol. 38:586-590.

89. Loeffler J., H. Habart, U. Brauchle, U. Schumacher and H. Einsele. 2000. Comparison between plasma and whole blood specimens for detection of *Aspergillus* by PCR. J. Clin. Microbiol., 38:3830-3833.

90. Lortholary O., M.C. Mejohas, B. Dupont, J. Cadranel, D. Salmon-Ceron, D. Peyramond, D. Simonin. 1993. Invasive aspergillosis in patients with acquired immunodeficiency syndrome: report of 33 cases. Am. J. Med., 95:177-187

91. Machetti M. , M. Feasi, N. Mordini, B. Van Lint, J.P. Latgé, J. Sarfati and C. Viscoli. 1998. Comparison of an enzyme immunoassay and a latex agglutination system for the diagnosis of invasive aspergillosis in bone marrow transplant recipients. Bone Marrow transplant, 31:917-921.

92. Madan T.P., P. Eggleton, U. Kishore, P. Strong, S. Aggrawal, P.U. Sarma, and K. B. Reid. 1997. Binding of pulmonary surfactant proteins A and D to Aspergillus fumigatus conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages. Infect. Immun., 65:3171-3179.

93. Maertens J., J. Verhagen, H. Demuynck, P. Brock, G. Verhoef, P. Vandenberghe, J. Van Eldere, J. Verbist and M. Bogaerts. 1999. Autopsy controlled prospective evaluation of serial screening circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive aspergillosis. J. Clin. Microbiol., 37:3223-3228.

94. Manavathu E.K., J. Cutright, D. Loebenberg, P. Chandrasekhar. 2000. A comparative study of the *in vitro* susceptibilities of clinical and laboratory selected resistant isolates of *Aspergillus* spp. To amphotericin B, itraconazole, voriconazole and posaconazole (SCH 56592). J. Antimicrob. Chemother., 46:229-234.

95. Manso E., M. Montillo, G. De Sio, S. D'Amico, G. Discepoli, and P. leoni. 1994. Value of antigen and antibody detection in the serological diagnosis of invasive aspergillosis in patients with haematological malignancies. Eur. J. Clin. Microbiol. Infect.Dis., 30:756-760.

96. Margulis L., K.V. Schwartz. 1988. Five kingdoms, 2nd edn, Freeman and Co., New-York, 1-376.

97. Martin P.J., J.A. Hansen, R. storb, E.D. Thomas. 1985. A clinical trial of *in vitro* depletion of T cells in donor marrow for prevention of acute graft-versus-host disease. Transplant. Proc. 17:486-487.

98. Matthews R., J.P. Burnie, A. Fox and S. tabaqchali. 1985. immunoblot analysis of serological responses in invasive aspergillosis. J. Clin. Microbiol, 38:1300-103.

99. Mayer J.M., Nimer L., Carroll K. 1992. Isolated pulmonary aspergillar infection in cardiac transplant recipients: case report and review. Clin. Infect. Dis., 15:698-700.

100. Melchers, W.J.G., P.E. Verweij, P. van den Hurk, A. van Belkum, B. De Pauw, J. Hoogkamp-Korstanje, and J.F.G.M. Meis. 1994. General primer-mediated PCR for detection of *Aspergillus* species. J. Clin. Microbiol. 32:1710-1717.

101. Meletiadis J., J.F. Meis, J.W. Mouton, J.P. Donnelly, P.E. Verweij. Comparison of NCCLS and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) methods of *in vitro* susceptibility testing of filamentous fungi and development of a new simplified method. 2000. J. Clin. Microbiol., 38:2949-2954.

102. Meyohas M.C., P. Roux, J.L. Poirot, J.L. Meynard, J. Frottier. 1994. Aspergillose au cours du syndrome d'immunodéficience acquise. Pathol. Biol., 42:6447-651.

103. Micheli P. 1729. Nova plantarum genera juxta turnefortu methodum disposita, Florence 234.

104. Milosev B., Davidson C.M., Gentles J.C., Sandison A.T. 1966. Aspergillosis of the paranasal sinuses and orbit in northern Sudanese. Lancet, 1:746-747.

105. Minamoto G.Y., T.F. Barlam, and N.J. 1992. Invasive aspergillosis in patients with AIDS. Clin. Infect. Dis., 14 :66-74.

106. Moore C., C. Walls, D. Denning. 2000. In vitro activity of the new triazole BMS-207147 against *Aspergillus* species in comparison with itraconazole and amphotericin B. Antimicrob. Agents Chemother., 44: 441-443.

107. Mosquera J., P.A. Warn, J. Morrissey, C.B. Moore, C. Gil-Lamaignere, D.W. Denning. 2001. Susceptibility testing of *Aspergillus flavus:* inoculum dependence with itraconazole and lack of correlation between susceptibility to amphotericin B *in vitro* and outcome *in vivo*. Antimicrob. Agents Chemother., 45:1456-1462.

108. Müllbacher A. and R. D. Eichner. 1984. Immunosuppression *in vitro* by a metabolite of a human pathogenic fungus. Proc. Natl. Acad. Sci. USA, 81:3835-3837.

109. Müllbacher A., P. Waring and R. D. Eichner. 1985. Identification of an agent in cultures of *Aspergillus fumigatus* displaying anti-phagocytic and immuno-modulating activity *in vitro*.J. Gen. Microbiol., 131:1251-1258.

110. Mullis K.B., F. Faloona, S. Scharf , R. Saiki, G. Horn, H.A. Erlich. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol., 51:263-273.

111. Mullis K.B., F. Faloona; 1987. Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. Methods Enzymol., 155:335-350.

112. Nakao A., M. Yasui, T. Kawagoe, H. tamura, S. Tanaka, and H. Takagi. 12997. false positive endotoxemia derives from gauze glucan after hepatectomy for hepatocellular carcinoma with cirrhosis. Hepatogastroenterology, 44:1413-1418.

113. Nash G., R. Irvine, R.L. Kerschmann and B. Herndier. 1997. Pulmonary aspergillosis in acquired pulmonary deficiency syndrome: autopsy study of an emerging pulmonary complication of human immunodeficiency virus infection. Human pathology, 28:1268-1275.

114. National Committee for Clinical Laboratory Standards. 1997. reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Proposed standard M38-P. National Committee for clinical Laboratory Standards, Wayne, Pa.

115. Nolard N. Risques d'aspergillose invasive et contamination de l'environnement. 1994. Pathologie Biologie, 42 :706-710.

116. Obayashi T., M. Yoshida ,D. H. Tamura, J. Aketagawa, S. tanaka and T. Kawai. 1992. determination of plasma β1-3glucan: a new diagnosis aid to deep mycosis. J. med. Vet. Mycol., 30:275-280.

117. Odds F.C., F. Van Gerven, A. Espinel-Ingroff, M.S. Barlett, M.A. Ghannoum, M.V. Lancaster, M.A. Pfaller, J.H. Rex, M.G. Rinaldi, T.J. Walsh. 1998. Evaluation of possible

correlations between antifungal susceptibilities of filamentous fungi *in vitro* and antifungal treatment in animal infection models. Antimicrob. Agents Chemother., 42:282-288.

118. Odds F., M. Oris, P. Van Dorsselaer, F. van Gerven. 2000. Activities of an intraveinous formlulation of itraconazole in expérimental disseminated *Aspergillus*, *Candida*, and *Cryptococcus* infections. Antimicrob. Agents Chemother., 44:3180-3183.

119. Overdijk B.G., J. Van Stijn and F. Odds. 1996. Chitinase levels in guinea pigs blood are increased after systemic infection by *Aspergillus fumigatus*. Glycobiology, 6:627-634.

120. Paris S.E., E. Boisvieux-Ulrich, B. Crestani, O. Oucine, D. Taramelli, L. Lombardi, and J.P. Latgé. 1997. Internalization of *Aspergillus fumigatus* conidia by epithelial and endothelial cells. Infect. Immun., 65:1510-1514.

121. Patel R. and C. Paya. 1997. Infections in solid-organ recipients. Clin. Microbiol. Rev., 10:86-124.

122. Patterson T.F., P. Miniter, patterson J.E., J.M. Rappeport and V. T. Andriole. Aspergillosis antigen detection in the diagnosis of invasive aspergillosis. 1995. J. Infect. Dis., 171:1553-1558.

123. Persing D.H., T.F. Smith, F.C. Tenover and T.J. White. 1993. Diagnostic molecular microbiology. Principles and applications. American society for microbiology, Washington.

124. Peterson D.E. and S.C. Schrimpf. 1989. *Aspergillus* sinusitis in neutropenic patients with cancer: a review. Biomed. Pharmacother., 43:307-312.

125. Petheram E.S., R.M.E. Seal. 176. Aspergillus prosthetis valve endocarditis. Thorax, 31:380-390.

126. Piens M.A., J. Troncy, M.C. Nicolle, A. Mercatello, D. Fiere, M. Mojon. Aspergilloses invasives chez des patients atteints d'hémopathies malignes. 1993. A propos de 29 observations. J. Mycol. Medic., 3:79-83.

127. Potter M. 2001. European experience with oral solution and intraveinous itraconazole. Oncology (Huntingt), 15 (11 suppl 9):27-32.

128. Raper K.B. eand D.I. Thom. 1965. The genus *Aspergillus*, William and Wilkins Co, Baltimore M.A., 686.

129. Reddy, L.V., A. Kumar, and V.P. Kurup. 1993. Specific amplification of *Aspergillus fumigatus* DNA by polymerase chain reaction. Moll. Cell. Probes 7:121-126.

130. Reichard U., S. Büttner, H. Eiffert, F. Staib and R. Rüchel. 1990. Purification and characterisation of an extracellular serine protease from *Aspergillus fumigatus* and its detection in tissue. J. Med. Microbiol., 33:243-251.

131. Richardson M.D. *Aspergillus* and *Penicillium* species. Chap 16, 281-312. *In* Microbiology and microbial infections. Topley and Wilson's. Volume 4. Medical mycology. L. Ajello & R.J. Hay (eds), 9th ed., London, 1998.

132. Rohrlich P., J. Sarfati, P. Mariani, M. Duval, A. Carol, C. saint-Martin, E. bingen, J.P. Latgé and E. Vilmer. 1996. Prospective sandwich-linked immunosorbent assay for serum galactomannan: early predictive value and clinical use in invasive aspergillosis. Pediatr. Infect. Dis., 15:232-237.

133. Roilides E., K. Uhlig, D. Venzon, P.A. Pizzo, and T.J. Walsh. 1993. Enhancement of oxidative response and damage caused by human neutrophils to *Aspergillus fumigatus* hyphae by granulocyte-stimulating factor and γinterferon. Infect. Immun., 61:1185-1193.

134. Roilides E., T. Robinson, T. Sein, P. Pizzo, T.J. Walsh. 1994. *In vitro* et *ex vivo* effects of cyclosporin A on phagocytic host defences against *Aspergillus fumigatus.* Antimicrob. Agents Chemother. 38:2883-2888.

135. Saiki, R.K., S.J. Scharf , F. Faloona, K.B. Mullis, G.T. Horn, and H.A. Erlich. 1985. Enzymatic amplificationof ß-globin sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

136. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich. 1988. primer-directed amplification of DNA with a thermostable DNA probes. Science, 239:487-491.

137. Schaechter M. Biology of infectious agents. p.19-61. *Dans* Mechanisms of microbial disease, third edition. M. Schaechter, N. Cary Engelberg, B. I. Eisenstein, G. Medoff (eds), Lippincott Williams & Wilkins, 1999, Baltimore, USA.

138. Schaffner A. 1984. Acquired immune deficiency syndrome: is disseminated aspergillosis predictive of underlying cellular immune deficiency? J. Infect. Dis., 149 :822-829.

139. Schaffner A. 1985. Therapeutic concentrations of of glucocorticoïds suppress the antimicrobial activity of human macrophages without impairing their responsivness to γ-interferon. J. Clin. Microbiol., 76:1755-1764.

140. Schaffner A. 1989. Experimental basis for the clinical epidemiology of fungal infections. A review. Mycoses. 32:499-515.

141. Scharf S.J., G.T. Horn, H.A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science, 233:1076-1078.

142. Segretain J. 1970. Les moisissures de l'air de Paris. Bull. Soc. Fr. Myc. Med.. 18:7.

143. Serody J.S. and T.C. Shea. 1997. Prevention of infections in bone marrow transplant recipients. Inf. Dis. Clin. North America, 11:459-477.

144. Skladny, H., D. Buchheidt, C. Baust, F. Krieg-Schneider, W. Seifarth, C. Leib-Mosch, and R. Hehlmann. 1999. Specific detection of *Aspergillus* species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. J. Clin. Microbiol. 37:3865-3871.

145. Simmonds E.J., Littlehood J.M., V. Hopwood, E.G.V. Evans. 1994. *Aspergillus fumigatus* colonisation and population density of place of residence in cystic fibrosis. Arch, Dis. Child, 70, 139-140.

146. Spreadbury, C., D. Holden, A. Aufauvre-Brown, B. Brainbridge, and J. Cohen. 1993. Detection of *Aspergillus fumigatus* by polymerase chain reaction. J. Clin. Microbiol. 31: 615-621.

147. Staib F., M. Seibold, M. Grosse. 1989. *Aspergillus* findings in AIDS patients suffering from cryptococcosis. Mycosis, 32:516-523.

148. Storm G and E. Van Eltern. Biopharmaceutical aspects of lipids formulations of Amphotericin B. 1997. Eur. J. Clin. Microbiol. Infect. Dis., 16:64-73.

149. Staples C.A., E.Y. Kang, J.L. Wright, P. Philips, N.L. Muller. 1995. Invasive pulmonary aspergillosis in AIDS: radiographic, CT, and pathologic findings. Radiology, 196:409-414.

150. Sturtevant J. and J.P. Latgé. 1992. Participation of complement in the phagocytosis of the conidia od *A. fumigatus* by human polymorphonuclear cells. J. Infect. Dis., 166:580-586.

151. Stynen D., A. Goris, J. Sarfati, and J.P. Latgé. 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol., 33:497-500.

152. Sulahian A., M. Tabouret, P. ribaud, J. Sarfati, E. Gluckman, J.P. Latgé and F. Derouin. 1996. Comparison of an enzyme immunoassay and latex agglutination test for the detection of galactomannan in the diagnosis of invasive aspergillosis. Eur. J. Clin. Microbiol. Infect. Dis., 15:139-145.

153. Sullivan K.M. 1987. Immunoglobulin therapy in bone marrow transplantation. Am.L J. Med., 83:34-45.

154. Symoens F., S. Bertout, M.A. Piens, J. Burnod, F. Renaud, N. Nolard, F. Chapuis, R. Grillot, EBGA Network. 2001. A longitudinal study of lung transplant recipients infected with *Aspergillus*: genetic polymorphism of *A. fumigatus*. Transpl. infect., 20:970-978.

155. Tabone M. D., H. Vu-Thien, J.P. Latgé., J. Landman-Parker, P. Perez-Castignoli, D. Moissenet and G. Leverger. 1997. Value of galactomannan detection by sandwich enzyme linked immnosorbent assay in the diagnosis and follow-up of invasive aspergillosis. Opport. Pathog., 9:7-13.

156. Talbot G.H., A. Huang and M. Provencher. 1991. Invasive *Aspergillus* rhinosinusitis in patients with acute leukaemia. Rev. Infect. Dis., 13:219-232.

157. Tang C.M., J. Cohen and D.W. Holden. 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol., 6:1663-1671.

158. Tang C.M., D.W. Holden, A. Aufauvre Brown, J. Cohen. 1993. The detection of *Aspergillus spp.* by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am. Rev. Respir. Dis., 148:1313-1317.

159. Tang C.M., J. Cohen, T. Krausz, S. Van Noorden and D.W. Holden. 1993. the alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of the invasive pulmonary aspergillosis. Infect. Immun., 61:1650-1656.

160. Thau N., N. Monod, B. Crestani, C. Rolland, G. Tronchin, J.P. Latgé and S. Paris. 1994. Rodletness mutants of *Aspergillus fumigatus*. Infect. Immun., 62:4380-4388.

161. Tomee J.F.C., G.P.M. Mannes, W. Van der Bij, T.S. Van der werf, W.J. De Boer, G.H. Koëter, H.F. Kauffman. 1996. Serodiagnosis and monitoring of *Aspergillus* infection after lung transplantation. Ann. Intern. Med., 125:197-201.

162. Trull A.K., J. Parker, R.E. Warren. 1985. IgG enzyme linked immunosorbent assay for diagnosis of invasive aspergillosis: retrospectice study over 15 years of transplant patients. J. Clin. Pathol., 38:1045-1051.

163. Van Zutphen L.F.M., V. Baumans, A.C. Beynen. 1993. Principles of laboratory animal science. A contribution to the humane use and care of animals and to the quality of experimental results. Elsevier (Ed), Amsterdam, the Netherlands.

164. Verweij, P.E., J.F. Meis, , P. Van den Hurk, B. De Pauw, J.A.A. Hoogkamp-Korstanje, and W.J. Melchers. 1994. Polymerase chain reaction as a diagnostic tool for invasive aspergillosis: evaluation in bronchoalveolar lavage fluid from low risk patients. Serodiagn. Immunother. Infect. Dis., 6:203-208.

165. Verweij, P.E., D. Stynen, A.J.M. M. Rijs, B.E. De Pauw, J.A.A. Hoogkamp-Korstanje, and J.F.G.M. Meis. 1995. Sandwich enzyme linked immunosorbent assay compared with Pastorex latex agglutination test for diagnosing invasive aspergillosis in immunocompromised patients. J. Clin. Microbiol. 33:1912-1914.

166. Verweij, P.E., J.P. Latgé, A.J.M. M. Rijs, W.J. Melchers, B.E. De Pauw, J.A.A. Hoogkamp-Korstanje, and J.F.G.M. Meis. 1995. Comparison of antigen detection and PCR assay using bronchoalveolar lavage fluid for diagnosing invasive pulmonary aspergillosis in patients receiving treatment for haematological malignancies. J. Clin. Microbiol., 33, 3150-3153.

167. Verweij P.E., E.C. Dompeling, J.P. Donnelly, A.V. Schattenberg, and J.F. Meis. 1997. Serial monitoring of *Aspergillus* antigen in the early diagnosis of invasive aspergillosis with two examples. Infection, 25:86-89.

168. Verweij P.E., Z. Erjavec, W. Sluiters, W. Goessens, M. Rozenberg-Arska, Y.J. Debets-Ossenkopp, H.F. Guiot and J.F. Meis. 1998. Detection of antigen in sera of patients with invasive aspergillosis: intra- and inter-laboratory reproducibility. J. Clin. Microbiol., 36:1612-1616.

169. Vogeser M., A. Haas, D. Aust and G. Ruckdeshel. 1997. Postmortem analysis of invasive aspergillosis in a tertiary care hospital. Eur. J. Clin. Microbiol. Infect. Did., 16:1-6.

170. Wallace J.M., R. Lim, B.L. Browdy, P. Hopewell, J. Glassroth, M; Rosen, L. Reichman, P. Kvale and Pulmonary complication of HIV group. 1998. Risk factors and outcomes associated with identification of *Aspergillus* on respiratory specimens from persons with HIV diseases. Chest, 114:131-137.

171. Walsh T.J. 1990. Invasive pulmonary aspergillosis in patients with neoplasic diseases. Sem. in Resp. Inf. 5:111-122.

172. Walsh T.J. and B.H. Bulkey. 1982. *Aspergillus* pericarditis in the immunocompromised patient: a clinicao-pathologic study. Cancer, 5:111-122.

173. Walsh T.J., J. Karp and P.A. Pizzo. 1996. Fungal infections of the respiratory tract. p. 235-268. *In* Kibbler C.C., D.W.R. Mackenzie and F.C. Odds (ed.), Principles and practice of clinical mycology, John Wiley & Sons Ltd.

174. Williamson E., J. Leeming, H. Palmer, C. Steward and D. Warnock. 2000. Diagnosis of invasive aspergillosis in bone marrow transplant recipients by polymerase chain reaction. Br. J. Haematol., 108:132-139.

175. Whittaker R.H. 1969. New concepts of kingdoms of organisms. Science, 163: 150-163.

176. Witteveen C.F., M. Venhuis and J. Visser. 1992. Lacalization of glucose and catalase activies in *Aspergillus niger*. Appl. Environ. Microbiol., 58:1190-1194.

177. Yamakami, Y., A. Hashimoto, I. Tokymatsu, and M. Nasu. 1996. PCR detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. J. Clin. Microbiol. 34: 2464-2468.

178. Yamakami, Y., A. Hashimoto, E. Yamagata, Kamberi P., Karashima R., Nagai H., and M. Nasu. 1998. Evaluation of PCR for detection DNA specific for *Aspergillus* species in sera of patients with various forms of pulmonary aspergillosis. J. Clin. Microbiol. 36:3619-3623.

179. Young R.C., J.E. Bennet. Invasive asperegillosis: absence of antibody response. 1971. Am. Rev. Resp. Dis., 104:710-716.

ANNEXES

ANNEXE 1 : Valeurs des CMI des 4 antifongiques vis à vis des 106 souches d'*Aspergillus fumigatus*.

Tableau A. Valeurs des CMI des 106 souches d'*A. fumigatus* vis à vis des 4 antifongiques testés.

	Amphotéricine B		**Itraconazole**		**Voriconazole**		**Posaconazole**	
	0,03125 à 16 µg/ml		*0,03125 à 16 µg/ml*		*0,03125 à 16 µg/ml*		*0,004 à 16 µg/ml*	
	Lecture visuelle	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*
N° souche	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*
1	*1*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
2	*1*	*1*	*0,25*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
3	*0,25*	*0,5*	*0,25*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
4	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
5	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
6	*1*	*0,5*	*0,25*	*0,5*	*0,25*	*0,5*	*0,03125*	*0,03125*
7	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
8	*1*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,01563*	*0,01563*
9	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
10	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,03125*	*0,03125*
11	*0,25*	*0,25*	*0,25*	*0,25*	*0,0625*	*0,0625*	*0,01563*	*0,01563*
12	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,5*	*0,01563*	*0,01563*
13	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,03125*
14	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,03125*	*0,0625*
15	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,01325*
16	*2*	*2*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
17	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
18	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
19	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
20	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,03125*
21	*1*	*1*	*0,5*	*0,5*	*0,125*	*0,25*	*0,03125*	*0,0625*
22	*1*	*1*	*1*	*1*	*0,125*	*0,125*	*0,03125*	*0,03125*
23	*2*	*2*	*0,25*	*0,25*	*0,25*	*0,25*	*0,03125*	*0,03125*
24	*1*	*1*	*0,5*	*0,5*	*0,5*	*0,25*	*0,01563*	*0,01563*
25	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,03125*	*0,03125*
26	*0,5*	*0,5*	*0,25*	*0,25*	*1*	*1*	*0,03125*	*0,0625*
27	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,03125*	*0,03125*
28	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
29	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,03125*
30	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
31	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
32	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,5*	*0,01563*	*0,01563*
33	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
34	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,5*	*0,03125*	*0,03125*

N° de souche en vert: environnement; N° en rouge: invasion; N° en noir: colonisation.

Tableau A. Valeurs des CMI des 106 souches d'*A. fumigatus* vis à vis des 4 antifongiques testés.

	Amphotéricine B		**Itraconazole**		**Voriconazole**		**Posaconazole**	
	0,03125 à 16 µg/ml		*0,03125 à 16 µg/ml*		*0,03125 à 16 µg/ml*		*0,004 à 16 µg/ml*	
	Lecture visuelle	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*
N° souche	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*
	CMI (µg/ml)	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*
35	*2*	*2*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
36	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
37	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
38	*1*	*2*	*0,5*	*0,5*	*0,5*	*0,5*	*0,01563*	*0,01563*
39	*0,5*	*0,5*	*0,5*	*0,25*	*0,5*	*0,5*	*0,03125*	*0,03125*
40	*1*	*1*	*0,5*	*0,5*	*1*	*1*	*0,03125*	*0,03125*
41	*0,25*	*0,25*	*0,5*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
42	*1*	*1*	*1*	*1*	*0,25*	*0,25*	*0,03125*	*0,03125*
43	*1*	*1*	*0,125*	*0,25*	*0,5*	*1*	*0,03125*	*0,03125*
44	*0,25*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
45	*0,5*	*0,5*	*0,25*	*0,125*	*0,25*	*0,25*	*0,01563*	*0,01563*
46	*0,5*	*0,5*	*0,25*	*0,25*	*0,5*	*0,5*	*0,01563*	*0,01563*
47	*0,25*	*0,25*	*0,25*	*0,25*	*0,5*	*0,5*	*0,03125*	*0,03125*
48	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
49	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
50	*0,5*		*0,25*	*0,125*	*0,25*	*0,125*	*0,0625*	*0,0625*
51	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
52	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
53	*1*	*1*	*1*	*1*	*0,25*	*0,25*	*0,01563*	*0,03125*
54	*0,5*	*0,5*	*0,0625*	*0,0625*	*0,25*	*0,25*	*0,01563*	*0,01563*
55	*0,25*	*0,25*	*0,25*	*0,25*	*0,125*	*0,0625*	*0,03125*	*0,03125*
56	*1*	*1*	*0,25*	*0,25*	*0,25*	*0,25*	*0,125*	*0,125*
57	*0,5*	*0,5*	*0,25*	*0,25*	*0,5*	*0,5*	*0,03125*	*0,03125*
58	*1*	*1*	*0,5*	*1*	*0,25*	*0,25*	*0,01563*	*0,01563*
59	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
60	*1*	*0,5*	*1*	*1*	*0,5*	*0,5*	*0,03125*	*0,0625*
61	*0,5*	*0,5*	*0,25*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
62	*1*	*0,5*	*0,25*	*0,25*	*0,5*	*0,5*	*0,03125*	*0,03125*
63	*1*	*1*	*0,5*	*0,5*	*0,125*	*0,125*	*0,01563*	*0,01563*
64	1	0,5	0,5	0,5	0,25	0,25	0,0312	0,0312
65	1	0,5	0,25	0,25	0,125	0,125	0,0156	0,0156
66	1	1	0,5	0,5	0,5	0,5	0,0156	0,0156
67	*1*	*1*	*0,5*	*1*	*0,5*	*0,5*	*0,01563*	*0,01563*

N° de souche en vert: environnement; N° en rouge: invasion; N° en noir: colonisation.

Tableau A. Valeurs des CMI des 106 souches d'*A. fumigatus* vis à vis des 4 antifongiques testés.

	Amphotéricine B		**Itraconazole**		**Voriconazole**		**Posaconazole**	
	0,03125 à 16 µg/ml		*0,03125 à 16 µg/ml*		*0,03125 à 16 µg/ml*		*0,004 à 16 µg/ml*	
	Lecture visuelle	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*
N° souche	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*	*48 H CMI (µg/ml)*
68	*1*	*1*	*1*	*1*	*0,25*	*0,25*	*0,0078*	*0,0078*
69	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,01563*	*0,01563*
70	*1*	*1*	*1*	*1*	*0,25*	*0,25*	*0,01563*	*0,01563*
71	*0,25*	*0,25*	*0,25*	*0,25*	*0,125*	*0,125*	*0,0078*	*0,0078*
72	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,0078*	*0,01563*
73	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,01563*	*0,0156*
74	*1*	*2*	*0,25*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
75	*1*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,03125*	*0,03125*
76	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,01563*	*0,03125*
77	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,5*	*0,03125*	*0,03125*
78	*1*	*1*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
79	*1*	*1*	*1*	*0,5*	*0,125*	*0,125*	*0,01563*	*0,01563*
80	*1*	*1*	*0,125*	*0,125*	*0,25*	*0,25*	*0,01563*	*0,01563*
81	*1*	*1*	*0,5*	*0,5*	*0,5*	*0,5*	*0,01563*	*0,01563*
82	*1*	*1*	*0,125*	*0,25*	*0,25*	*0,25*	*0,03125*	*0,01563*
83	*0,5*	*0,5*	*1*	*1*	*0,5*	*0,5*	*0,03125*	*0,03125*
84	*1*	*1*	*1*	*1*	*0,5*	*0,5*	*0,03125*	*0,03125*
85	*0,5*	*0,5*	*0,25*	*0,25*	*0,125*	*0,25*	*0,03125*	*0,01563*
86	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,03125*	*0,03125*
87	*0,5*	*0,5*	*0,125*	*0,125*	*0,125*	*0,125*	*0,0078*	*0,0078*
88	*0,5*	*0,5*	*0,25*	*0,5*	*0,25*	*0,25*	*0,0078*	*0,0078*
89	*1*	*1*	*0,25*	*0,5*	*0,125*	*0,25*	*0,0078*	*0,0078*
90	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,0078*	*0,0078*
91	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,0078*	*0,0078*
92	*1*	*0,5*	*0,25*	*0,5*	*0,25*	*0,25*	*0,0078*	*0,0078*
93	*0,25*	*0,25*	*1*	*1*	*0,5*	*0,5*	*0,01563*	*0,01563*
94	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
95	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
96	*0,25*	*0,125*	*0,5*	*0,5*	*0,5*	*0,5*	*0,125*	*0,125*
97	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,03125*
98	*0,5*	*0,5*	*0,5*	*0,5*	*0,125*	*0,25*	*0,0078*	*0,0078*
99	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,01563*	*0,01563*
100	*0,25*	*0,25*	*0,25*	*0,25*	*0,125*	*0,25*	*0,03125*	*0,03125*
101	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,01563*	*0,01563*

N° de souche en vert: environnement; N° en rouge: invasion; N° en noir: colonisation.

Tableau A. Valeurs des CMI des 106 souches d'*A. fumigatus* vis à vis des 4 antifongiques testés.

	Amphotéricine B		**Itraconazole**		**Voriconazole**		**Posaconazole**	
	0,03125 à 16 µg/ml		*0,03125 à 16 µg/ml*		*0,03125 à 16 µg/ml*		*0,004 à 16 µg/ml*	
	Lecture visuelle	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*	*Lecture visuelle*	*630 nm*
N° souche	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*	*48 H*
	CMI (µg/ml)	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*	*CMI (µg/ml)*
102	*0,5*	*0,5*	*0,5*	*0,5*	*0,25*	*0,25*	*0,03125*	*0,01563*
103	*0,5*	*0,5*	*0,125*	*0,125*	*0,125*	*0,125*	*0,01563*	*0,01563*
104	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,125*	*0,01563*	*0,01563*
105	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,5*	*0,03125*	*0,03125*
106	*0,5*	*0,5*	*0,25*	*0,25*	*0,25*	*0,25*	*0,0078*	*0,0078*
moyenne des 106 souches	**0,666693**	**0,642561**	**0,374985**	**0,387448**	**0,270408**	**0,283073**	**0,020028**	**0,020797**

N° de souche en vert: environnement; N° en rouge: invasion; N° en noir: colonisation.

ANNEXE 2 :Lettre d'approbation du COMITE D'ETHIQUE.

Université de Liège
Centre Animalier Universitaire
Commission d'éthique

Liège, le 21 juin 2001

Dr. HAYETTE
Microbiologie Médicale
Tour de Pathologie - B23

Cher (ère) Collègue,

Votre dossier intitulé " Diagnostic d'aspergillose pulmonaire invasive chez le lapin: antigénémie versus Polymerase chain reaction (PCR)" a été examiné par la Commission d'Ethique en date du 6 avril 2001 et a reçu le numéro 51.

La Commission a émis un avis favorable .

Nous vous prions de croire, Cher (ère) Collègue, en l'expression de nos sentiments les meilleurs.

Professeur B. NICKS.
Président de la Commission d'Ethique.

ANNEXE 3 : Article publié dans une revue internationale

(Journal of Clinical Microbiology)

URNAL OF CLINICAL MICROBIOLOGY, June 2001, p. 2338–2340 Vol. 39, No. 6
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Detection of *Aspergillus* Species DNA by PCR in Bronchoalveolar Lavage Fluid

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The usefulness of a nested PCR assay for detection of *Aspergillus* sp. DNA was evaluated in 177 bronchoalveolar lavage (BAL) fluid specimens. This test was accurate both to diagnose culture-negative BAL fluid specimens from patients with invasive pulmonary aspergillosis and to confirm culture-positive samples. However, it did not differentiate between infection and colonization.

Diagnosis in the early stages of invasive pulmonary aspergil- sis (IPA) is very difficult, as clinical and radiological signs are onspecific and the sensitivity of fungal cultures is low (40 to 0%), even when combined with direct microscopic examina- on (2, 5). Circulating-antigen detection may contribute to the iagnosis. However, up to 8% false positives are reported (11). ther antigens are under investigation as diagnostic tools (6), ut attention has now turned to molecular methods. The role f PCR assay of bronchoalveolar lavage (BAL) fluid for diag- osing IPA does not clearly emerge in the literature (1, 3, 7, 9, 0, 14). Therefore, we have evaluated a nested-PCR-based mplification of *Aspergillus fumigatus* DNA that targets the enes encoding alkaline proteases of the fungus to determine ie role of PCR in diagnosing IPA from BAL fluid under utine conditions.

Clinical and reference strains were tested to assess the spec- icity of the method: *A. fumigatus* (three strains), *Aspergillus avus* (three strains), *Aspergillus niger* (two strains), *Aspergillus idulans* (two strains), *Aspergillus terreus, Aspergillus glaucus, usarium oxysporum, Fusarium solani* (ATCC 10154), *Paecylo- iyces* spp., *Penicillium* spp., *Pseudallescheria boydii* (two rains), *Trichoderma harzianum* (two strains), *Rhizopus rhizo- odiformis, Mucor* spp., *Candida albicans* (ATCC 10231), and *andida glabrata* (ATCC 90030). The fungal strains were cul- vated on Sabouraud dextrose agar and incubated at 37 or 8°C for up to 5 days, depending on the species. The clinical olates were identified by macroscopic, microscopic, and cul- ire characteristics (12).

All patients undergoing bronchoscopy at the University Hos- ital of Liège (Liège, Belgium) during a 12-month period 1997 to 1998) were included in the study. There were 74 nmunosuppressed and 103 nonimmunosuppressed patients. atients were referred to as immunosuppressed if they were nder long-term corticotherapy for chronic obstructive pulmo- ary disease (COPD) (n = 18) or other diseases (n = 13) or if ey had hematological malignancy (n = 16), organ transplan- ition (n = 5), AIDS (n = 3), or cancer (n = 19). Nonimmunosuppressed patients had bronchoscopy for investigation of severe pneumonia. Medical, radiological, histopathological, and microbiological records and autopsy findings were reviewed to assess IPA. Three groups were defined (A, proven or probable aspergillosis [n = 10]; B, colonization [n = 5]; and C, no evidence of aspergillosis [n = 162]) according to the following criteria: proven, histology with hyphal tissue invasion and *Aspergillus*-positive culture of one or more respiratory specimens or of a lung biopsy; probable, *Aspergillus*-positive culture from two or more respiratory samples and positive clinical or radiological findings (pulmonary infiltrates, nodular opacity, cavitation, or persistent fever under broad-spectrum antimicrobial chemotherapy) or positive histology without *Aspergillus*-positive culture; colonization, *Aspergillus*-positive cultures from one or more respiratory samples without clinical or radiological evidence (see above) of respiratory tract infection due to this pathogen; and no evidence of aspergillosis, *Aspergillus*-negative cultures and no clinical or radiological findings (see above) of aspergillosis.

After reception in the laboratory, each BAL fluid specimen was homogenized and separated into two parts: one part (1 to 5 ml) was stored at −20°C until it was analyzed by PCR, and the second part was included in the routine procedure and tested for the presence of bacteria, yeasts, fungi, parasites, and viruses. A PCR assay was performed a minimum of 1 week after sampling, and the clinicians were unaware of the results. The technique used for DNA extraction from fungal culture was adapted from that of Tang et al. (13), except that DNA was resuspended in 30 μl of water containing RNase A at a concentration of 50 μg/ml. The same technique was used for DNA extraction from yeasts except for the culture, which was made on Sabouraud dextrose agar and incubated for 48 h at 37°C. DNA extraction from BAL fluid was adapted from techniques already published by Tang and colleagues (14) except for two details: (i) 200 μg of proteinase K (Sigma, St. Louis, Mo.) was added to the 500 μl of BAL fluid and buffer and (ii) DNA was dried in a dry-heating block and resuspended in 30 μl of distilled H_2O containing RNase A (50 μg/ml). Positive-displacement pipettes were used throughout, and DNA extraction buffer was extracted in parallel in order to preclude contamination.

We developed a nested PCR using as external primers alp 11

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TABLE 1. Clinical data and aspergillosis detection for group A (proven or probable aspergillosis) and group B (colonization) patients

Case no.	Underlying disease[a]	IPA group (disease[b])	BAL fungal culture	Autopsy performed	BAL PCR results[c]	Outcome[d]
roup A (n = 10)						
1	Hepatic transplantation	Proven (DIS)	*A. fumigatus*	Yes	+	Died
2	COPD (corticosteroid dependent)	Proven (CNA)	*A. fumigatus*	Yes	+	Died (AmB)
3	Chronic lymphoid leukemia + COPD	Probable	*A. fumigatus*	No	+	Died
4	RA (corticosteroid dependent)	Proven	Negative	Yes	+	Died
5	COPD (corticosteroid dependent)	Proven (DIS)	*A. fumigatus*	Yes	+	Died
6	Gastric carcinoma	Proven	*A. fumigatus*	Yes	+	Died
7	COPD (corticosteroid dependent)	Proven (CNA)	*A. fumigatus*	Yes	+	Died
8	Chronic myeloid leukemia blast-crisis	Probable	*A. fumigatus*	No	+	Alive (Vor)
9	Chronic lymphoid leukemia blast-crisis	Proven	Negative	Yes	+	Died
10	COPD (corticosteroid dependent)	Probable	*A. fumigatus*	No	+	Died
roup B (n = 5)						
1	Mitral valvulopathy	None	*A. fumigatus*	No	+	Alive (AmB/It)
2	COPD (corticosteroid dependent)	None	*A. fumigatus*	No	+	Died
3	COPD (corticosteroid dependent)	None	*A. fumigatus*	Yes	+	Died
4	COPD (no corticoid)	None	*A. fumigatus*	No	+	Alive
5	Myelodysplasia blast-crisis	None	*A. flavus*	Yes	+	Died

[a] RA, rheumatoid arthritis.
[b] DIS, disseminated aspergillosis; CNA, chronic necrotizing aspergillosis.
[c] +, positive.
[d] Vor, treatment with voriconazole; AmB/It, treatment with amphotericin B and itraconazole; AmB, treatment with amphotericin B.

5′-AGCACCGACTACATCTAC-3′) and alp 12 (5′-GAGAT GGTGTTGGTGGC-3′). These primers were derived from the equence of cloned fragments of genes encoding the alkaline roteases (Alp) of *A. fumigatus* and *A. flavus* (14). As internal rimers, we chose Alp13 (5′-CTGGCATACAACGCCGCTG-′) and Alp14 (5′-TTGTTGATCGCAACC-3′), expected to mplify a fragment of 527 bp. The primers were synthesized by Eurogentec, Liège, Belgium. The PCR mixtures were identical or both steps except for $MgCl_2$. They were carried out in a 0-μl volume containing 10 mM Tris-Cl at pH 8.3, 50 mM KCl, nd 1.5 mM $MgCl_2$ (2.25 mM for the second step), with 20 mol of both primers, 2.5 mM deoxynucleoside triphosphate buffer and deoxynucleoside triphosphate were provided by Takara, Otsu, Japan), and 1.25 U of *Taq* polymerase (Takara). A 5-μl volume of DNA was added to the mixture. Positive and negative controls were amplified in parallel to assess the validity of the procedure. Thermal cycling conditions (GeneAmp PCR system 2400; Perkin-Elmer Cetus, Norwalk, Conn.) were dentical for both PCRs: 5 min at 94°C; 30 cycles of 30 s at 94°C, 45 s at 63°C, and 2 min at 72°C; and a final extension step of 10 min at 72°C. For the nested PCR, 5 μl of the first mplified product was added to a new reaction mixture and mplified under the same conditions. The final amplified products were analyzed on 1.5% agarose gels stained with ethidium bromide and visualized by UV transillumination. Each sample was investigated for the presence of inhibitors by amplification of the β-globin gene (8).

No band of the expected size (527 bp) was detected with *A. niger, A. nidulans, A. glaucus, A. terreus*, or *A. flavus* or with the other fungal species. However, a band of 690 nm, corresponding to the sequence amplified by the first PCR, was observed for *A. flavus*. The sensitivity of the nested PCR was 25 fg of genomic DNA for *A. fumigatus* by ethidium bromide staining and 10 pg for *A. flavus*. Some BAL fluids (n = 5) were excluded because of lack of amplification of the β-globin gene. The PCR results are reported in Table 1. The sensitivity, specificity, and positive and negative predictive values of this PCR test for diagnosing IPA were 100, 96, 62, and 100%, respectively. All BAL fluids from patients with IPA were PCR positive. Those from the five cases of *Aspergillus* colonization were PCR and culture positive. In all, six PCR-positive cases were not associated with IPA among the 177 BAL fluid specimens, representing 3.4% false-positive results. Only one false-positive PCR result was induced in group C. Among the three patients with COPD and proven IPA (group A), two patients (cases 2 and 7) presented with chronic necrotizing aspergillosis and one (case 5) presented with disseminated aspergillosis. In two cases (4 and 9), the diagnosis of proven IPA was made at autopsy by histology and *A. fumigatus*-positive culture of a lung biopsy specimen.

In the present study, the PCR always confirmed the culture results, except for two culture-negative BAL fluid specimens, for which diagnosis of aspergillosis was missed and made at autopsy. In both cases, the PCR could have contributed to diagnosing IPA before death. Only one PCR-positive BAL result was observed for a patient with no evidence of aspergillosis: a burn patient who was intubated for 1 month. This false-positive result could be explained by contamination of the BAL fluid during sampling or the PCR process, or it could have been due to the colonization of the respiratory tract by *Aspergillus* spores during intubation. Some authors have reported up to 35% PCR-positive results for patients with no risk or low risk for aspergillosis and who did not develop IPA (1, 3, 9, 10, 14, 15). However, other studies performed in neutropenic patients (4) or in nonimmunosuppressed patients (7) reported no false-positive PCR results for BAL fluids. Among our population, only 3.4% positive PCR results not associated with IPA were observed. Half of the patients (3 out of 6) had COPD. These patients may have had a relatively high level of tracheobronchial colonization, which differs from patients with hematological malignancies, who may have had minimum involvement before developing severe invasive fungal infection.

our study, the major risk factor associated with IPA was rticotherapy associated with COPD. However, the major risk ctor for aspergillosis is known to be a prolonged neutropenia, counting for its high frequency in patients with acute leuke-ia (2). In fact, in our institution, patients with hematological alignancies benefit from a good follow-up and are often eated early in cases where there is a suspicion of infectious sease.

In conclusion, nested PCR of BAL fluid is an accurate test to agnose culture-negative patients with IPA, but it does not fferentiate between infection and colonization. It is an ap-ropriate method to exclude *Aspergillus* sp. infection in pa-ents at risk of IPA and should be included in routine labo-tory practice for this immunocompromised population.

REFERENCES

. **Bart-Delabesse, E., A. Marmorat Khuong, J. M. Costa, M. L. Dubreuil-Lemaire, and S. Bretagne.** 1996. Detection of *Aspergillus* DNA in bronchoalveolar lavage fluid of AIDS patients by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **15:**24–25.
. **Bodey, G. P., and S. Vartarian.** 1989. Aspergillosis. Eur. J. Clin. Microbiol. Infect. Dis. **8:**413–437.
. **Bretagne, S., J. M. Costa, A. Marmorat-Khuong, F. Poron, C. Cordonnier, M. Vidaud, and J. Fleury-Feith.** 1995. Detection of *Aspergillus* species DNA in bronchoalveolar lavage samples by competitive PCR. J. Clin. Microbiol. **33:**1164–1168.
. **Jones, M. E., A. J. Barnes, B. A. Oppenheim, P. Balagopal, G. R. Morgenstern, and J. H. Scarffe.** 1998. PCR-ELISA for the early diagnosis of invasive pulmonary *Aspergillus* infection in neutropenic patients. J. Clin. Pathol. **51:** 652–656.
5. **Kahn, F. W., J. M. Jones, and D. England.** 1985. The role of bronchoalveolar lavage in the diagnosis of invasive aspergillosis. Am. J. Clin. Pathol. **86:**518–523.
6. **Latgé, J. P.** 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. **12:**310–350.
7. **Melchers, W. J. G., P. E. Verweij, P. van den Hurk, A. van Belkum, B. De Pauw, J. Hoogkamp-Korstanje, and J. F. G. M. Meis.** 1994. General primer-mediated PCR for detection of *Aspergillus* species. J. Clin. Microbiol. **32:** 1710–1717.
8. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, and H. A. Erlich.** 1985. Enzymatic amplification of β-globin sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.
9. **Skladny, H., D. Buchheidt, C. Baust, F. Krieg-Schneider, W. Seifarth, C. Leib-Mosch, and R. Hehlmann.** 1999. Specific detection of *Aspergillus* species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. J. Clin. Microbiol. **37:**3865–3871.
10. **Spreadbury, C., D. Holden, A. Aufauvre-Brown, B. Bainbridge, and J. Cohen.** 1993. Detection of *Aspergillus fumigatus* by polymerase chain reaction. J. Clin. Microbiol. **31:**615–621.
11. **Stynen, D., A. Goris, J. Sarfati, and J. P. Latgé.** 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol. **33:**497–500.
12. **Sutton, D. A., A. W. Fothergill, and M. Rinaldi.** 1998. Guide to clinically significant fungi. The Williams & Wilkins Co., Baltimore, Md.
13. **Tang, C. M., J. Cohen, and D. W. Holden.** 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. **6:**1663–1671.
14. **Tang, C. M., D. W. Holden, A. Aufauvre-Brown, and J. Cohen.** 1993. The detection of *Aspergillus* spp. by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am. Rev. Respir. Dis. **148:**1313–1317.
15. **Verweij, P. E., J. F. G. M. Meis, P. van den Hurk, B. E. De Pauw, J. A. A. Hoogkamp-Korstanje, and W. J. G. Melchers.** 1994. Polymerase chain reaction as a diagnostic tool for invasive aspergillosis: evaluation in bronchoalveolar lavage fluid from low risk patients. Serodiagn. Immunother. Infect. Dis. **6:**203–208.

ANNEXE 4 : Posters présentés dans un congrès international

ICAAC (Intersciences conference on antimicrobial agents and chemotherapy)

ICAAC 1999

ICAAC 2001

PCR in Bronchoalveolar Lavage Fluid

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Introduction

Aspergillus fumigatus is an opportunistic nosocomial pathogen which causes severe to fatal infection in neutropenic patients. The risk of invasive aspergillosis (IA) increases with the duration of neutropenia and can reach 70% after 5 weeks of neutropenia (1).

The diagnostic of IA is one of the most difficult anf the gold standard is still based on culture and/or histology results which sensitivity is very low. Recently, molecular biology methods were developped to diagnose aspergillosis in urines, serum, blood and BAL.

We used a nested PCR-based amplification of *A. fumigatus* DNA in BAL fluids that targets the genes-encoding alkaline proteases of the fungus to evaluate the usefulness of PCR to diagnose IA.

Abstract

The usefulness of a nested PCR for detection of *Aspergillus fumigatus* DNA was evaluated in bronchoalveolar lavage (BAL) fluid during a period of two years (1996-1998). The aim of the study was to assess the role of PCR in diagnosing invasive pulmonary aspergillosis (IPA). Methods: a nested PCR-based amplification of fragments of genes-encoding alkaline proteases from Aspergillus fumigatus was used to testt 167 BAL samples. All samples were checked for the absence of amplification inhibitors. Medical, radiological, microbiological records and autopsy findings were reviewed for assessing invasive aspergillosis. All successive patients investigated by BAL were included in the study. They were distributed in three groups: A: proven or probable aspergillosis (n=11),; B, colonization (n=6); C: no evidence of IPA (n=154). PCR results were compared to culture detection as gold standard and to clinical data. Results: BAL fluids from 10 patients of group A were PCR positive. One case was falsely negative. Among group B, one case was PCR positive, and the second one PCR negative but had negative BAL cultures (only culture positive sputum). No false positive was detected among group C. Comparing to culture, sensitivity was 91%, specificity, 100%, positive predictive value, 100%, and negative predictive value, 99%. Conclusions: Aspergillus fumigatus PCR in BAL fluid was an accurate test to diagnose culture negative patients with IPA and to confirm culture positive samples; however it does't make difference between infection and colonization. 2. It is an appropriate test to exclude *Aspergillus* infection in patients at risk of invasive illness.

Methods

•Clinical data: all patients undergoing bronchoscopy at the university hospital of Liege between 1996 and 1998 were included in the study and distributed in three groups:
- •A: proven or probable aspergillosis (n=11)
- •B:colonization (n=2)
- •C: no evidence of aspergillosis (n=154)

•BAL fluid specimens: all lavages collected by the microbiological laboratory were included in the study and stored at -20°C before processing. Microbiological examination was directly performed on every sample and results recorded.

•DNA extraction: adapted from Tang et coll. (2).
- •BAL fluid (250µl) + extraction buffer (250µl) containing proteinase K (100 µg): 65°C for 60 mn
- •extraction once with phenol:chloroform:isoamylalcool (25:24:1) and then with chloroform:isoamylalcool (24:1).
- •Precipitation of DNA by ethanol

•Nested PCR: The target DNA corresponds to genes-encoding alkaline proteases from *A. fumigatus* (2).
External primers: alp11 and alp12 (2).
Internal primers: alp13 (5'-CTGGCATACAACGCCGCT-3')
alp 14 (5'-TTGTTGATCGCAACC-3')
Product length after amplification: 527 base pairs.
Mix, (50µl volume): 10mM TRIS-Cl at pH 8,3, 50 mM KCl and 1.5 mM MgCl2 with 100 pmol of both primers and 1.25 U Taq polymerase (Takara Taq, Japan).
Thermal cycling conditions: 30 cycles at 94°C for 30 sec., 63°C for 45sec., and 72°C for 2 min.
Products of PCR : analyzed on 1.5% agarose gels, stained with ethidium bromide and visualized by UV transillumination.

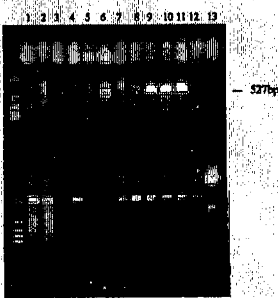

Figure 1. Amplification products on ethidium stained agarose gel. Above: lanes: 1-5, 7,8: negative BAL. Lanes 6, 8: patient 7 from group A. Lane :10,11: *A. fumigatus* DNA. Lanes 12, 13: negative sample (water). Below: beta-globine amplification of BAL samples to assess the lack of amplification inhibitors.

Results

case	UNDERLYING DISEASE	IPA	CULTURE positive	HISTOLOGY positive	PCR positive	SURVIVAL
GROUP A: API or probable API (n=11)						
1	Hepatic transplantation	yes	yes	yes	yes	no
2	COPB corticoids dependent	yes	yes	yes	yes	no
3	Hematologic malignancy	yes	yes	no histology	yes	no
4	Cardiac transplantation	yes	yes	yes	yes	no
5	Arthritis	yes	no	yes	yes	no
6	COPB corticoids dependent	yes	yes	yes	yes	no
7	Gastric carcinoma	yes	yes	yes	yes	no
8	COPB corticoids dependent	Probable	yes	yes	no	no
9	Bone marrow transplantation	yes	yes	no histology	yes	no
10	Hematologic malignancy	yes	yes	yes	yes	no
11	Hematologic malignancy	yes	yes	no histology	yes	no

case	UNDERLYING DISEASE	IPA	CULTURE positive	HISTOLOGY positive	PCR positive	SURVIVAL
GROUP B: colonization (n=2)						
1	Mitral valvulopathy	no	yes	no histology	yes	yes
2	COPB (no corticoids)	no	yes	no histology	no	yes

COPB: Chronic obstructive pulmonary bronchitis.

n	UNDERLYING DISEASE	IPA	CULTURE positive (n)	HISTOLOGY positive (n)	PCR positive (n)
GROUP C: no evidence of aspergillosis (n=154)					
154	Respiratory disease	0	0	0	0

Sensitivity and specificity of PCR

	PCR-	PCR+	
IPA	1	10	11
NO IPA	155	1	156
	156	11	167

Sensitivity: 91%
Specificity: 100%
Predictive values
negative 99%
positive 100%

Conclusions

1.*A. fumigatus* PCR in BAL fluid was an accurate test to diagnose culture negative patients with IPA and to confirm culture positive samples.
However it doesn't make difference between infection and colonization.

2. This PCR is an appropriate method to exclude *Aspergillus* infection in patients at risk of invasive illness.

Bibliography

•1.Ribaud P., Esperou-Bourdeau H., Devergie A., Gluckman E. Aspergillose invasive et allogreffe de moelle. Path. Biol., 1994, 42, 652-655.
•2.Tang C., Holden D., Aufauvre-Brown, Cohen J. The detection of Aspergillus spp. by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am. Rev. Resp. Dis., 1993, 148, 1313-1317.

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Comparative *in vitro* activity of amphotericine B, itraconazole, voriconazole and posaconazole against *Aspergillus fumigatus*.

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Abstract

Background. New azoles have been successfully used as treatment of invasive aspergillosis. The purpose of this study was to compare the *in vitro* activity of posaconazole (Posa) with that of amphotericin B (AmB), itraconazole (Itra) and voriconazole (Vor) against *A. fumigatus* isolates according to NCCLS method (M38-P), and to compare visual and spectrophotometric readings for MIC determination. **Methods.** A total of 106 *A. fumigatus* isolates were selected as follows: 81 clinical isolates from colonized patients, 18 from patients with invasive aspergillosis and 7 environmental isolates. Their *in vitro* susceptibility was evaluated by the NCCLS microdilution method (M38-P) in RPMI 1640 medium. Determination of results was made by visual and spectrophotometric readings (630 nm) after 48 hours incubation at 35°C. Three *A. fumigatus* reference strains (IHEM 5734, 6149 and 13935) were included as control.
Results. 1. Geometric mean MICs/MIC_{90} (µg/ml) obtained by visual reading were respectively 0.66/1 (AmB), 0.37/0.5 (Itra), 0.27/0.5 (Vor) and 0.02/0.03 (Posa). 2. MIC values were comparable by spectrophotometric and by visual readings for all antifungal agents tested (p>.05) and did not depend on the isolate's origin (p>.05). 3. Posaconazole had the lowest MICs (p< 0.001). 4. The itraconazole-resistant reference strain did not give cross resistance with voriconazole and posaconazole. **Conclusion:** Among azoles, posaconazole had a better *in vitro* activity against *A. fumigatus* than did voriconazole or itraconazole. Spectrophotometric reading could replace the less standardized visual reading for NCCLS microdilution method and MIC values obtained were comparable among all *A. fumigatus* isolates.

Introduction

New azoles have been recently introduced in the treatment of invasive aspergillosis. Clinical studies are in progress to compare the efficiency and toxicity of these molecules to the gold standard, amphotericin B.

The purpose of our study was first to determine the MICs of two new azoles, voriconazole (Vor) and posaconazole (posa), against *Aspergillus fumigatus* isolates from diverse origins and to compare the results to amphotericin B (AmB) and Itraconazole (Itra) MIC values.

Secondly, the aim of the study was to compare the visual reading with a spectrophoto-metric one in order to enhance the standardisation of the method. E-test was also performed for posaconazole and compared with NCCLS method in a parallel direction.

Materials and methods

Strains: 106 isolates of *A. fumigatus* have been selected as follows: 81 clinical isolates from colonized patients, 18 from patients with invasive aspergillosis and 7 environmental isolates. Three *A. fumigatus* (IHEM 5734, 6149 and 13935) reference strains have been included as controls.
Culture: the strains have been cultivated on Sabouraud dextrose agar following the recommandations of the NCCLS M38-P microdilution methodology (1).
NCCLS method: RPMI 1640 medium (Gibco BRL, Life Technologies, USA) buffered with MOPS (Sigma, St Louis, USA) has been used. All antifungals were dissolved in DMSO before diluting them in the RPMI medium. The concentrations tested were as follows: 0.031 to 16 µg/ml for AmB, Itra and Vor; 0.004 to 16 µg/ml for Posa. The drugs were kindly provided as pure powder by their manufacturers: AmB (Bristol-Myers Squibb, USA), Itra (Janssen Pharmaceutica, Beerse, Belgium), Vor (Pfizer, New-York, USA), and Posa (Schering Plough, Kenilworth, USA). Determining MICs: reading has been performed according to the NCCLS recommandations (visual reading) with a score 0 for AmB and score 2 for the azoles. A spectrophoto-metric reading at 630 nm was also performed and compared to the visual reading with the same scoring system.
E-test posaconazole: E-test (AB-biodisk, Solna, Sweden) for posaconazole was performed on RPMI 1640 buffered agar (1.5%). Reading was performed after 24h incubation at 35°C.

Results

MICs geometric means (µg/ml) of *A. fumigatus* isolates (n=106).

Amphotericin B		Itraconazole		Voriconazole		Posaconazole		
Visual	630 nm	Visual	630 nm	Visual	630 nm	Vis.	630n	Etest
0.67	0.64	0.37	0.39	0.27	0.28	0.02	0.02	0.01

1. There is no significative difference between visual and spectrophotometric readings (p > 0.05).
2. Among azoles, posaconazole has lower MICs than voriconazole and itraconazole (p < 0.001).
3. E-test and NCCLS reults for posaconazole are in agreement (p>0.05).

MICs geometric means (µg/ml) by visual reading

Origin of isolates	Amphotericin B	Itraconazole	Voriconazole	Posaconazole
Environmental (n=7)	0.74	0.37	0.25	0.02
Colonising (n=81)	0.61	0.43	0.43	0.02
Invasive (n=18)	0.67	0.36	0.27	0.02

3. There is no significative difference according to origin of isolates (p > 0.05) .

Discussion and conclusions

The standardisation of the NCCLS M38-P method can be improved by use of spectrophotometric reading. This method avoids the variability due to the reader giving a better reproducibility. However, automatic MICs calculation is needed to spead up the procedure.

Antifungal susceptibility is not a marqueur of pathogenicity for *A. fumigatus* since there is no significative difference between isolates MICs from different origins. Since authors have reported *A. fumigatus* itraconazole-resistant isolates (2), it seems useful to test the susceptibiility before treatment particularly for azoles.

Among azoles posaconazole has the lowest MICs values. It is a very promising drug and the undergoing clinical trials will determine if the drug can successfully cure invasive aspergillosis without serious side effects.

Nevertheless the NCCLS method could better be replaced by an easier method for routine laboratory. According to our study and to others authors (3), E-test method could be used for *A. fumigatus* as this methodology gives good correlations with NCCLS method .

References

1.National Committee for clinical Laboratory standards. 1998. Reference method for broth dilution antifungal susceptibility of conidium-forming filamentous fungi. Proposed standards M38-P. National Committee for clinical laboratory standards, Wayne, Pa.

2.Denning D. et al. 1997. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother., 41, 1364-1368.

3.Szekely A. and al. 1999. Comparison of E-test and broth microdilution methods for antifungal drug susceptibility testing of molds. J. Clin. Microbiol., 37, 1480-1483.

ANNEXE 5 : Formules de tampons

Bleu de bromothymol

Bleu de bromothymol 250mg

Ficoll 15ml

H2O QSP100ml

Dissoudre le bleu dans l'eau puis ajouter la glycérol. Mélanger une nuit par agitateur magnétique. Conserver à 4°C. A utiliser tel quel en ajoutant 2µl de bleu à 10 µl de produit amplifié.

Bromure d'éthidium (solution stock).

Bromure d'éthidium .... 1 g

H2O qsp .... 100ml.

Mélanger jusqu'à dissolution complète. Conserver à température ambiante et à l'abri de la lumière.

EDTA 0.5mM

Disodium éthylène diaminetetracétate-2H2O ....186.12 g

H2Oppi ....qsp 1l

Ajuster à pH 8 avec NaOH (soit environ 20g) et ajuster le volume à 1 litre.

N.B. Utiliser un agitateur magnétique pour dissoudre la poudre. Le sel d'EDTA ne devient soluble que lorsque le pH est ajusté à 8.

Marqueurs de poids moléculaire (Eurogentec, Belgium).

Marqueur IV (ΦX174 /Hae III) 75µg

Marqueur IV 150µl

Bleu de Bromothymol 120µl Concentration finale du marqueur 0.1µg/µl

H2O ppi 480ml

Marqueur V (ΦX174 /Hinc II digest) 75µg

Marqueur V 150µl

Bleu de Bromothymol .... 120µl   Concentration finale du marqueur 0.1µg/µl

H2O ppi 480µl

Tampon d'extraction selon Tang et coll. pour extraction d'*Aspergillus sp.* dans les LBA.

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200 mM Tris-HCl pH8 .... soit 2.42g

0.5M NaCl .... soit 2.92g

0.01M EDTA .... soit 0.37g

1% SDS .... soit 1 ml de solution à 10%

H2O ppi QSP100ml

Préparer en tube Falcon de 50ml. Stériliser sur filtre 0.2µ.

Tampon d'extraction selon Yamakami et coll. pour extraction d'*Aspergillus sp.* dans le sang.

100mM KCl .... soit 0,745 g

20mM Tris-HCl (pH 8.3) .... soit 24.22 mg

5mM MgCl2 .... soit 0.5 ml d'une solution à 1 M (Sigma Aldrich).

0.9% Tween 20 .... soit 900µl

0.2 mg/ml de gélatine .... soit 20mg

H2O ppi qsp100ml

Filtrer sur filtre 0.2µ.

Tampon de lyticase

50mM Tris pH 7.5 .... soit 0.302 mg

1 mM EDTA .... soit 0.015 mg

20% β-mercaptoéthanol 97µl

H2O ppi .... qsp 50 ml

Filtrer sur filter 0.2 µ.

TBE 5x concentré (Tampon tris-Acide borique-EDTA)

Tris base .... 54 g

Acide borique .... 27.4 g

EDTA à 0.5mM pH 8 .... 20ml

H2O ppi .... qsp 1l

Conserver à température ambiante.

ANNEXE 6 : Protocoles d'extraction de l'ADN

1. Technique modifiée de Yamakami et coll. ().

La composition du tampon de lyse est donnée dans les annexes « tampons ».
Cette technique consiste à mélanger 100 µl d'échantillon avec 100 µl de tampon. La protéinase K est ajoutée extemporanément à la concentration de 200 µg (à la place de 60µg/ml recommandée par les auteurs) soit 20 µl d'une solution à 10 µg/µl. Le mélange est incubé 60 minutes à 65°C puis 30 minutes à 95°C et centrifugé à 12.000 g pendant 10 minutes à +4°C.
Le surnageant est utilisé pour réalisation de la PCR.

2.Technique d'extraction sur colonne QIAamp DNA mini kit, tissue protocole (QIAamp DNA Mini Kit, QIAgen, Allemagne).

1.Ajouter 180 µl de solution ATL au culot de leucocytes et 20 µl de protéinase K (Qiagen). Mélanger par vortex et incuber 3 heures à 55°C.

2. Ajouter 200µl de solution AL, mélanger par vortex et incuber 10 min. à 70°C. Centrifuger quelques secondes pour concentrer tout le liquide dans le fond du tube.

3. Ajouter 200 µm d'éthanol, bien mélanger par vortex. Centrifuger quelques secondes pour concentrer tout le liquide dans le fond du tube.

4. Placer une colonne dans un tube de 2 ml (Qiagen), puis déposer tout le liquide sur la colonne. Fermer le bouchon. Centrifuger 1 min à 8000 rpm.

5. Sortir la colonne du tube et la placer dans un nouveau tube de 2 ml. Déposer 500 µl de solution AW1 (Qiagen). Centrifuger 1 min à 8000 rpm.

6. Sortir la colonne du tube et la placer dans un nouveau tube de 2 ml. Déposer 500 µl de solution AW2 (Qiagen). Centrifuger 1 min à 14.000 rpm.

7. Sortir la colonne du tube recentrifuger 1 min à 14.000 pour éliminer toute trace de AW2.

8. Sortir la colonne du tube. Ajouter 50 µl de solution AE et laisser 5min à température ambiante. Centrifuger 1 min à 14.000 rpm et garder l'éluat. Celui-ci peut être congelé à –20°C ou traité directement en PCR.

3.Techique Amplicor® Whole Blood (Roche).

Le kit Amplicor® Whole Blood est composé de deux réactifs :

BLD WS permet de lyser les globules rouges et d'isoler les leucocytes à partir du sang. Il s'agit d'une solution de phosphate de sodium contenant moins de 0.4% de détergent et de 0.05 % d'azide de sodium.

BLD EXT permet la lyse des leucocytes. Il s'agit d'un tampon Tris-HCl contenant 0.01% de protéinase K, 7.5 mg de $MgCl_2$, 1% de détergent et 0.05% d'azide sodium.

Technique de lyse des globules rouges :

Le sang total prélevé sur EDTA a été soumis à une lyse des globules rouges par le kit de préparation des échantillons Amplicor® Sang Total (Roche diagnostics, Brandburg, USA), permettant de lyser les globules rouges et blancs en deux étapes séparées et grâce à deux réactifs différents.

1. On mélange 1 volume (500 µl) de sang total à 2 volumes (1 ml) de solution de lyse BLD WS. On mélange 10 à 15 fois par retournement, puis on laisse à température ambiante pendant 5 minutes pour faciliter la lyse. Ensuite, les tubes sont centrifugés pendant 3 minutes à vitesse maximale (15.000 trs/min). Le culot de globules blancs se distingue par sa couleur plus claire. Le surnageant est prélevé et éliminé.
2. Deux volumes de solution de lavage sont ajoutés au culot. On mélange sur Vortex pour remettre en suspension et on mélange de nouveau par retournement 10 à 15 fois. Puis on centrifuge comme précédemment 3 minutes à vitesse maximale (15.000 tr/min).

3. L'étape 2 est répétée encore une fois de façon à éliminer au maximum l'hémoglobine présente dans le prélèvement.

Le culot peut être directement traité pour extraction de l'ADN ou être congelé à –80°C.

Technique de lyse du culot leucocytaire :

Le culot cellulaire est décongelé à température ambiante quelques minutes s'il n'a pu être traité directement.

La méthodologie consiste à ajouter 200 µl de réactif BLD EXT au culot décongelé et de placer le tube fermé au bain marie à 60°C pendant 45 minutes. Le tube est ensuite maintenu à 100°C pendant 30 minutes pour éliminer complètement la protéinase K.

L'échantillon est ensuite centrifugé quelques secondes, pour concentrer l'échantillon au fond du tube. On peut le garder à +4°C jusqu'à la réalisation de la PCR.

4.Technique d'extraction par TriPure.

(TriPure isolation reagent®, Boehringer Manheim, Allemagne).

1. Ajouter 1 ml de TriPure à 100 µl de solution à extraire. Incuber 5 min à température ambiante.

2. Ajouter 0.2 ml de chloroforme (0.2 ml par ml de TriPure). Mélanger vigoureusement par vortex. Incuber 15 min à température ambiante.

Centrifuger 15 min à 12.000 g à 4°C.

On obtient trois phases :
-phase supérieure aqueuse (incolore) contenant le RNA.
-interface contenant le DNA de couleur blanche.
-phase inférieure organique (rouge) contenant les protéines.

3. Eliminer la phase supérieure et ajouter 0.3 ml d'éthanol pur pour séparer DNA et protéines (soit 0.3 ml d'éthanol pur par ml de Tripure). Mélanger par inversion et incuber 3 minutes à T°C ambiante.

4. Centrifuger 5 min à 2000g à 4°C. Eliminer le surnageant (protéines).

5. Laver le culot avec une solution de citrate de sodium 0.1M contenant 10 % d'éthanol (1ml de solution par ml de TriPure). Incuber 30 min à température ambiante en mélangeant de temps en temps.

6. Centrifuger 5 min à 2000g à 4°C. Eliminer le surnageant.

7. Répéter l'étape de lavage deux fois.

8. Laver le culot dans une solution d'éthanol à 75% (1.5 à 2 ml par ml de TriPure). Incuber 20 min à T°C ambiante en mélangeant de temps en temps.
Centrifuger 5 min à 2000g à 4°C et éliminer le surnageant.

9. Sécher le culot (dans un bloc chauffant). Dissoudre le culot dans de l'eau. Conserver à 4°C ou congeler à –20°C jusqu'à réalisation de la PCR.

ANNEXE 7 : Milieux de culture

RPMI 1640 (NCCLS M38-P)

Dissoudre 10.4 g de RPMI 1640 (avec glutamine, et sans bicarbonate)(Difco) dans 900 ml d'eau distillée.

Ajouter 2 g de $NaHCO_3$ (Sigma) et 34.53g de tampon MOPS (Sigma). Ajuster le pH à 7 à l'aide de NaOH 1N. Compléter avec de l'eau pour atteindre un volume final d'un litre.

Filtrer la solution (filtres Nalgène, Nalge Nunc corporation, New York, USA).

Conserver à +4 °C au maximum un mois.

SABOURAUD DEXTROSE AGAR (Oxoid)

Sabouraud dextrose agar 65g

Eau distillée QSP.................................... 1litre

Peser la poudre et compléter à 1 litre avec de l'eau distillée. Placer sur un barreau magnétique dans le récipient et placer sur un agitateur magnétique. Agiter jusqu'à formation d'une suspension homogène. Chauffer sur un bec bunzen en agitant fréquemment puis distribuer dans les tubes selon les quantités désirées. Autoclaver 30 minutes à 120°C, puis sortir les tubes encore chauds les incliner et les laisser refroidir. Boucher complètement une fois refroidis et conserver à température ambiante.

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