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BRIEF COMMUNICATION

A SIMPLE PCR-BASED PROCEDURE FOR PLAGUE DIAGNOSIS

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SUMMARY

Supernatant of boiled spleen saline-suspensions of *Yersinia pestis* experimentally infected animals were used as template for PCR amplification without DNA extraction. PCR sensitivity was enhanced by a second round of amplification (Nested). No amplification was observed from non-infected animals.

KEYWORDS: Plague; *Yersinia pestis*; Rodents; PCR; Diagnosis.

Yersinia pestis, the causative agent of plague, is widely distributed throughout the world in endemic foci involving a diversity of mammalian hosts and flea vectors[1,8]. Most human cases of plague result from the bite of infected fleas associated with wild rodents. Thus, laboratory testing to detect *Y. pestis* infection in such fleas and rodents has been a primary component of health programs for surveillance and control of plague. In the present communication a PCR assay is described that may be useful in conjunction with more conventional laboratory tests, to monitor the control of plague.

Although PCR-based procedures proved very useful for the diagnosis of a number of infectious diseases, its use in the diagnosis of plague has been mostly directed to the detection of DNA of *Y. pestis* in experimentally infected fleas and *Y. pestis* strains from collections. In addition, all the described procedures are time consuming as they involve DNA extraction[3,5,6].

We used supernatants of boiled spleen saline-suspensions of *Y. pestis* experimentally infected animals as template for PCR amplification without previous DNA extraction. Four (4) Swiss-Webster mice and four (4) *Calomys callosus*, obtained from the animal facilities of the CPqAM/FIOCRUZ/MS, were infected with *Y. pestis* strain PB 881 (CPqAM/FIOCRUZ/MS collection). *C. callosus* is a plague-susceptible rodent usually found in Brazilian plague foci. Animal infection resulted from the bite of infected fleas, a situation very close to the natural infection. Autopsies and *Y. pestis* identification in the inoculated animals were carried out by standard techniques[2]. One normal, non infected animal of each species was tested as negative control. Two sets of primers for nested PCR were designed, based on the sequence of the *caf1* gene of the pFra (90 kb), *Y. pestis* specific plasmid, coding for the F1 protein[4]. The primers were synthesized by the British Bio Technology Products Ltd. Oxon, UK. One aliquot of 100 µl of saline suspensions of spleen homogenates were boiled for 10 minutes in a water-bath. Ten µl of the boiled supernatant was directly used as template for PCR amplification with the outer primers, targeted to an external sequence (5' - CGG GAA TTC GAG GTA ATA TAT GAA AAA AAT CA3' and 5' - CCG CTG CAG ATT ATT GGT TAG ATA CGG-3'). The total reaction mix (25 µl) including DNA template, consisted of 10 pmol of the outer primers, 2 mM dNTP, 1.5 mM $MgCl_2$ and 1U of *Taq* DNA Polymerase (Cenbiot, RS, BR). PCR was carried out in 25 cycles (94°C for 1 min.; 50°C for 2 min.; 72°C for 3 min), and a final step of 72° C for 7 min. The reactions were performed in a DNA thermal cycler (Perkin-Elmer). After amplification, 5 µl

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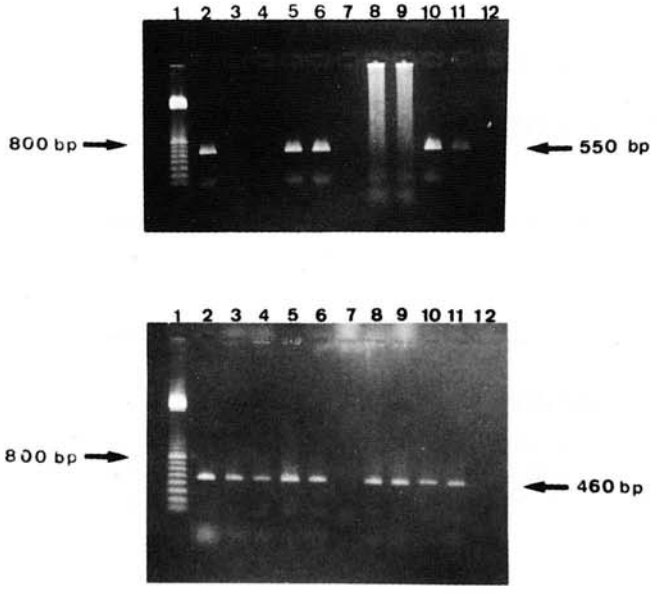

Fig. 1: Agarose gel analysis of PCR products with the outer primers in A and the inner primers in B. Lanes: 1, 100 base-pair lader DNA, molecular weight markers; 2, *Y. pestis* DNA; 3-6, infected mice; 7, non infected mice; 8-11, infected *C. callosus*; 12, non infected *C. callosus*.

of the PCR products were electrophoretically separated on 1% agarose gels, in TBE buffer. The amplified DNA fragments were visualized by ethidium bromide staining and UV transillumination. Samples from two out of four infected animals of each species were amplified. No amplification was observed either from the other two infected-animals of each species or from samples of non-infected animals. Figure 1-A shows the amplification of a DNA fragment of the expected size (550 bp) using the outer primers for the samples from two (2) mice and two (2) *C. callosus*.

All test and control samples that failed to yield PCR fragments on the first round were used in a second round of amplification following the same protocol except for a different set of primers (inner primers) targeted to an internal sequence (5'-TTG GAA CTA TTG CAA CTG CTA-3' and 5' - TTA GAT ACG GTT ACG GTT A-3'). Five µl of the PCR product was used as a template for the second PCR (Nested). The samples from infected animals that were not amplified in the first round of PCR, were amplified in the second round. Figure 1-B shows the amplification of a DNA fragment of 460 bp with the inner primers. As expected spleen samples from non-infected animals were not amplified by *Y. pestis* specific primers.

Blood and tissue components contain PCR inhibitors, that could interfere with the reaction. Furthermore the target DNA can be entrapped by organic materials reducing its accessibility to DNA polymerase[7,9] This is the reason why DNA extraction from biological samples is capable of removing some of the inhibitors and increase the sensitivity of PCR assays[6,7,9]. In spite of all these concerns spleen homogenates were directly used in our nested PCR assay with success. Possibly, if a step to remove inhibitors had been added the sensitivity would be increased but the assay would be more time consuming.

In conclusion, an easy, fast and reliable test allowing *Y. pestis* detection was devised and may be useful for plague control. The procedure was based upon PCR amplification directly from spleens of rodents followed by reamplification (Nested-PCR) which improves the sensitivity of the test. The whole PCR assay, including sample preparation, amplification and agarose gel analysis takes approximately 5 hours. If reamplification is required, additional 4 hours are required. The two-step procedure can be carried out in a working day. The technique described herein will expand the repertoire of tools for plague diagnosis.

RESUMO

Um método simples para o diagnóstico de peste por PCR

Triturados de baços de animais infectados experimentalmente com *Y.pestis*, suspensos em salina foram fervidos e os sobrenadantes usados diretamente para amplificação do PCR sem prévia extração do DNA. O limiar de detecção pode ser aumentado por uma segunda etapa de amplificação (Nested-PCR). Não houve amplificação a partir das amostras dos animais não infectados usados como controle.

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REFERENCES

1. ALMEIDA, A.M.P.; LEAL, N.C.; CARVALHO, F.G.; DANTAS SOBRINHO, J. & ALMEIDA, C.R. - Plague surveillance in Brazil: 1983-1992. **Rev. Inst. Med. trop. S. Paulo. 37:** 511-516, 1995.

2. BAHMANYAR, M. & CAVANAUGH, D.C. - Plague manual. Geneva, WHO, 1976.

3. CAMPBELL, J.; LOWE, J.; WALZ, S. & EZZEL, J. - Rapid and specific identification of *Yersinia pestis* by using a nested polymerase chain reaction procedure. **J. clin. Microbiol., 3:** 758-759, 1993.

4. GALYOV, E.E.; SMIRNOV, O.Y.; KARLISHEV, A.V. et al. - Nucleotide sequence of the *Yersinia pestis* gene encoding F1 antigen and the primary strucutre of protein. **FEBS Letters, 277:** 230-232, 1990.

5. HINNEBUSCH, J. & SCHWAN, T.G. - New method for plague surveillance using polymerase chain reaction to detect *Yersinia pestis* in fleas. **J. clin. Microbiol., 31:** 1511-1514, 1993.

6. NORKINA, O.V.; KULICHENKO, A.N.; GINISBURG, A.L. et al. - Development of a diagnostic test for *Yersinia pestis* by the polymerase chain reaction. **J. appl. Bact., 76:** 240-245, 1994.

7. OHHARA, M.; KUROSU, Y. & ESUMI, M. - Direct PCR of whole blood and hair shafts by microwave treatment. **Biotechniques, 17:** 726-728, 1994.

8. POLAND, J. & BARNES, A.M. - Plague. In: STEEL, J.H., ed. **CRC handbook series in zoonoses. Section A: bacterial, rickettsial and mycotic diseases**. Boca Raton, CRC Press, 1979.

9. ROSSEN, L.; NORRSKOV, K.H. & RASMUSSEN, O.F. - Inhibition of PCR by components of food samples, mibrobial diagnostic assays and DNA-extraction solutions. **Int. J. Food Microbiol., 17:** 37-45, 1992.

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