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DIAGNOSIS OF PLAGUE AND IDENTIFICATION OF VIRULENCE MARKERS IN *Yersinia pestis* BY MULTIPLEX-PCR

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SUMMARY

We have developed a procedure for the rapid diagnosis of plague that also allows the identification of prominent virulence markers of *Y. pestis* strains. This procedure is based upon the use of a single polymerase chain reaction with multiple pairs of primers directed at genes present in the three virulence plasmids as well as in the chromosomal pathogenicity island of the bacterium. The technique allowed the discrimination of strains which lacked one or more of the known pathogenic loci, using as template total DNA obtained from bacterial cultures and from simulated blood cultures containing diluted concentration of bacteria. It also proved effective in confirming the disease in a blood culture from a plague suspected patient. As the results are obtained in a few hours this technique will be useful in the methodology of the Plague Control Program.

KEYWORDS: *Yersinia pestis*; Plague; Blood culture; Virulence markers; Multiplex-PCR.

INTRODUCTION

Plague caused by *Yersinia pestis* is still a Public Health problem persisting in a number of natural foci in several countries worldwide[17, 21]. The speed of international travel constitutes a risk for the dissemination of the disease from the epidemic areas by infected travelers. A precise and quick diagnosis of plague would allow a prompt intervention during plague outbreaks[8]. Also, the knowledge of the characteristics of the strains in circulation in each focus is fundamental for the methodology of plague control.

In Brazil there are several natural plague foci distributed mainly in the Northeastern region, where the bacteria remain among the rodents and their fleas, occasionally reaching man[1,2,4]. Cycles of plague outbreaks in the Brazilian foci alternate with periods of quiescence of 5 to 10 years. In spite of the systematic bacteriological examination of rodents and fleas carried out uninterruptedly in all Brazilian foci, no *Y. pestis* isolation had been made in the last 11 years. During this period no human specimen has been submitted to bacteriological examination.

Bacteriological isolation of *Y. pestis* is the "gold standard" for plague diagnosis. The bacteria are identified by sensitivity to a specific bacteriophage and biochemical tests; inoculation of laboratory animals or the use of special culture media[5] are the usual methods to assess virulence properties of the isolates. All these procedures are too fastidious; culture and isolation of *Y. pestis* are not only time consuming but also expensive. Furthermore, inadequate procedures used for the shipment of the specimens from plague areas to the diagnosis centers may lead to desiccation, contamination of the samples or death of the bacteria.

Molecular techniques present several advantages over the traditional diagnostic procedures. Besides their speed, they are also particularly useful if the bacteria are no longer alive as their prior isolation is not required. Virulence genes located on three well-characterized plasmids (pFra, pYV and pPst) and on the chromosomic 'pathogenicity island' of *Y. pestis*[17] can be used as target for identification and characterization of this bacteria.

In this work we have developed a fast method for *Y. pestis* detection that also allows the identification of four virulence markers of the strains in a multiplex polymerase chain reaction. With the fortuitous occurrence of a human suspected plague case, on July 1997, at the Ibiapaba focus, we were able to show that this technique can be employed with success directly in patients' blood culture, without bacteria isolation and/or DNA extraction.

MATERIAL AND METHODS

Bacteria

Y. pestis P. PB 881 a typical virulent strain which harbors the three classical plasmids (pFra$^+$, pYV$^+$, pPst$^+$) and the *irp2* gene, a chromosomal pathogenicity marker; three atypical-mutant strains harboring the *irp2* gene but cured of one or two plasmids: YP 228/89 (pPst$^+$), YP 228/93

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Table 1
Primers used for PCR amplification of *Yersinia pestis* virulence genes

Primer pairs	Gene target	Size of PCR product	Location of the gene
5'- CGG GAA TTC GAG GTA ATA TAT GAA AAA AAT CA-3'	*caf*1	506 bp	Plasmid pFra
5'-CCG CTG CAG ATT ATT GGT TAG ATA CGG-3'			
5'- AGA GCC TAC GAA CAA AAC CCA C - 3'	*lcr*V	800 bp	Plasmid pYV
5'- GCA GGT GGT GGC AAA GTG AGA T- 3',			
5'- AAG TTC TAT TGT GGC AAC C -3'	*pla*	920 bp	Plasmid pPst
5'- GAA GCG ATA TTG CAG ACC -3',			
5'- AAG GAT TCG CTG TTA CCG GAC -3'	*irp*2	300 bp	Chromosome
5'- TCG TCG GGC AGC GTT TCT TCT -3'			

($pFra^+$, pYV^+) and YP 547 (pYV^+) and the vaccine Girard-Robic strain, EV76, harboring the three plasmids ($pFra^+$, pYV^+, $pPst^+$) but lacking *irp2*.

Human blood cultures

Two series of simulated blood cultures prepared inoculating four suspensions from $5x10^0$ to $5x10^3$ *Y. pestis* cells into flasks containing 25 ml of BHI and 2.5 ml of blood from two persons from non-endemic area.

One blood culture (25 ml of Brain Heart Infusion broth, BHI – Difco, inoculated with 2.5 ml of blood). The blood sample was obtained from a plague suspected patient, of the Ibiapaba Plateau plague focus in the State of Ceará, in July 1997.

Bacteriological procedures

The clinical sample was plated on Blood Agar Base (BAB – Difco) at 28 ºC for 48 h. The grow was tested for sensitivity to the antiplague bacteriophage and by biochemical tests as recommended by BAHMANYAR & CAVANAUGH[5] for isolation and identification of *Y. pestis.*

Preparation of samples for PCR

DNA from *Y. pestis* strains was extracted following the protocol described in SAMBROOK *et al.*[18]. Cells suspension in water of P. CE 882 was denatured by boiling and used in PCR reactions without DNA extraction.

The clinical and simulated blood cultures were used as template in PCR reactions, without DNA extraction. In order to remove PCR inhibitors they were processed following a protocol based in MAHBUBANI & BEJ[14]. 500 µl of each sample was centrifuged, washed once with 1 ml of TE (10 mM Tris-HCl; 1mM EDTA) pH 8.0, centrifuged again and the sediment resuspended in 100 µl of K buffer (50 mM KCl; 20 mM Tris-HCl pH 8.0; 2.5 mM $MgCl_2$). Lysis was performed by heating at 95 ºC for 10 minutes and freeze/thawing once. Ten µl of each sample was used as template in PCR reactions.

Primers

The primers were targeted at published sequences of the *caf1*, *pla* and *lcrV* genes located on the pFra, pPst and pYV plasmids respectively[9, 15,19] and the *irp2* chromosomal gene located on the 'pathogenicity island'[10]. The primer sequences, location of the genes and the sizes of the amplified fragments expected are described in Table 1.

Amplification

The reaction mixture, in a total volume of 25 µl contained: 50 mM KCl; 10 mM Tris-HCl (pH 8.0); 1.5 mM $MgCl_2$; 0.001 % gelatin; 200 µM of each dATP, dCTP, dGTP and dTTP (Pharmacia); 20 pmol of each primer; 1 U of Taq DNA Polymerase (CENBIOT, RS, BR).

The amplifications were performed in a DNA thermal cycler (Perkin-Elmer) programmed for 25 cycles composed of 1 minute at 94 ºC, 2 minutes at 50 ºC, 3 minutes at 72 ºC and a final step of 7 minutes at 72 ºC. After amplification, 10 µl of each product was submitted to electrophoresis in 1% agarose gels in TBE buffer, under constant voltage of 100 V. The gels were stained with ethidium bromide and photographed under ultraviolet light.

RESULTS

A multiplex-PCR technique, with a mixture of four primer pairs was tested, aiming the detection of *Y. pestis* on clinical samples and the characterization of virulence properties of *Y. pestis* strains. Primers were directed at genes encoding the most characteristics virulence factors of *Y. pestis*: the *caf1* gene, encoding the structural sub-unit of the F1 antigen[9], the gene *pla* encoding the coagulase and the plasminogen activator[19], the gene *lcrV* encoding the V antigen an immunogenic protein[15] and the *irp2* chromosomal gene[10] related to iron uptake.

Assays performed with total DNA from a typical *Y. pestis* strain (P. PB 881), produced four DNA fragments of the expected sizes: 300, 506, 800 and 920 base pairs (bp) corresponding respectively to *irp2, caf1, lcrV* and *pla* genes (Figure 1, lane 4). Accordingly, there was amplification of the three plasmidial genes with the strain EV76 whereas the segment corresponding to the *irp2* chromosomal gene was not produced (Figure

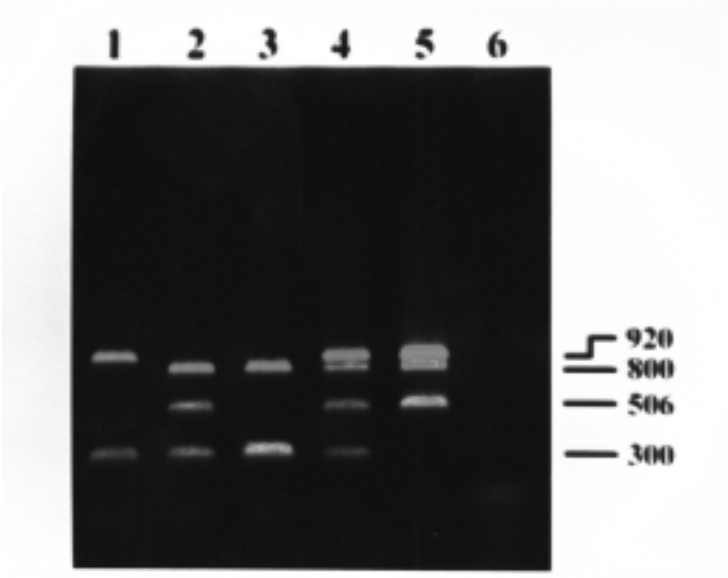

Fig. 1 - Products of amplification of the multiplex-PCR assay. Lines, 1: YP 228/89; 2: YP 228/93; 3: YP 547; 4: P.PB 881; 5: EV76.

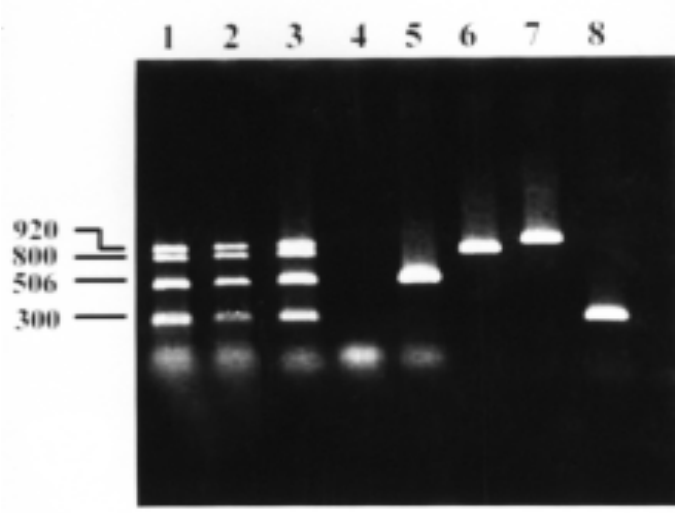

Fig. 2 - Products of amplification of the multiplex-PCR assay. Lines, 1: Processed blood culture; 2: Culture of P.CE 882 strain; 3: Purified DNA from P.CE 882 strain; 4: negative control (without DNA); 5-8: Amplifications from the processed blood culture with individual primers directed at the genes *caf1, lcrV, pla* and *irp2* respectively.

1, lane 5). With the plasmid-cured strains there was amplification compatible with the plasmid content of each strain and the *irp2* fragment was amplified with all of them (Figure 1, lanes 1-3).

Multiplex-PCR assays were also performed with two series of simulated blood cultures containing different concentrations of *Y. pestis*. A rapid and simple method, which removes PCR inhibitors without DNA extraction, was used to prepare blood cultures for PCR.

Bacteriological analysis of the blood sample taken from a plague suspected patient resulted on the isolation of a *Y. pestis* strain. The isolate, named P. CE 882, belongs to the "Oceanic" geographic variant: nitrate positive, glycerol negative[5] as most of the Brazilian strains[3,12]. This blood sample was also submitted to analysis by multiplex-PCR assay with the aim to evaluate the usefulness of this technique in *Y. pestis* diagnosis directly on clinical samples. Four DNA segments of the expected size were produced in the multiplex-PCR reaction (Figure 2, lane 1) confirming that this strain harbors the three typical plasmids and the pathogenicity island as do typical virulent strains. Results of PCR amplifications performed directly with the crude cells suspension of P. CE 882 culture or with the purified DNA were similar (Figure 2, lanes 2-3). There was also amplification of a DNA fragment of the expected size in the PCR reactions performed with each primer pair individually and with the processed blood culture (Figure 2, lanes 5-8).

DISCUSSION

PCR-based procedures has been developed for *Y. pestis* identification in experimentally infected fleas[11], mice and *Y. pestis* cultures from collection[6,16]. We have previously developed a quick and simple PCR-based procedure for *Y. pestis* identification directly from rodents´ spleens[13]. These procedures were directed at genes located on the plasmids pFra or pPst. However, *Y. pestis* strains lacking pPst occurs among rodents from the Caucasian plague foci[7]. As F1 negative *Y. pestis* strains have been isolated from rodents, fleas and man[22], it is likely that wild strains lacking the pFra plasmid may also exist in nature. The lack of the targeted gene would lead to false-negative results. The use of multiplex primers could overcome this handicap. Furthermore, it would allow the evaluation of the presence or absence of virulence markers in only one reaction. TSUKANO *et al*.[20] used a multiplex-primer system to detect *Y. pestis* among pathogenic *Yersinia* species and other enterobacteriaceae demonstrating that this technique may be useful for the detection and characterization of *Y. pestis* in contaminated samples.

Although it does not replace bacteriological isolation, the multiplex-PCR technique described here may be useful for the quick detection and characterization of *Y. pestis.* The amplification of four virulence genes of *Y. pestis* strains directly from the blood culture, provides a diagnosis faster than the traditional techniques. At the same time, it characterizes the virulence properties of the strain.

Though promising, the multiplex-PCR which was performed on a single sample obtained from only one patient's blood, does not guarantee its specificity and sensitivity, and the examination of other samples is sought after. It is likely that the multiplex-PCR technique directed at *Y. pestis* virulence markers will certainly be a practical tool for the plague surveillance programs among rodents and fleas. Therefore, studies are in progress in our laboratory in order to evaluate the usefulness of the multiplex-PCR in animal samples.

RESUMO

Diagnóstico da peste e identificação de marcadores de virulência de *Yersinia pestis* com Multiplex-PCR

Foi desenvolvido um procedimento para diagnóstico rápido da peste que ao mesmo tempo permite a identificação de marcadores de virulência de *Y. pestis*, com múltiplos iniciadores, em uma só reação de PCR. Foram usados quatro pares de iniciadores direcionados a genes presentes nos três plasmídeos de virulência e na ilha de patogenicidade dessa bactéria.

A técnica permitiu a discriminação de cepas que perderam um ou mais genes conhecidos, usando como molde DNA total obtido de culturas de *Y. pestis* e também de hemoculturas simuladas contendo concentrações conhecidas de *Y. pestis*. Também se mostrou eficiente para a confirmação da *Y. pestis* na cultura de sangue de um paciente suspeito de peste. Como os resultados são obtidos em poucas horas, esta técnica poderá ser útil na metodologia do Programa de Controle da Peste.

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