Abstract This study was designed to assess the efficacy of using oral washes (OWs) to diagnose Pneumocystis carinii pneumonia (PCP) in patients with a low parasite burden and to detect cases of subclinical infection. A total of 104 paired induced sputum (IS) samples and OWs from 104 HIV-seropositive patients and 32 OWs from immunocompetent healthy controls were studied. All of the control samples were negative. Fifty-two IS specimens were positive for Pneumocystis carinii, and 26 of these cases were also detected in the OWs using conventional stain or polymerase chain reaction. Twenty-four of the PCP cases had a high or a moderate parasite load and 28 had a low parasite load; among them, Pneumocystis carinii was detected in the OWs of 15 and 11 cases, respectively. Fifteen of the 104 IS samples studied belonged to patients who were asymptomatic carriers or who had a subclinical infection, and Pneumocystis carinii was detected in the OWs of 4 of these cases. The parasite was not detected in 37 IS samples and in 74 OWs. The results of this study indicate that in patients with a low pulmonary parasite burden, the number of organisms reaching the oral cavity is insufficient for reliable detection in OWs. Thus, OWs are less useful than IS samples for detecting Pneumocystis carinii in cases of pneumonia in which a low parasite burden and/or subclinical infection are present.

Introduction

Pneumocystis carinii is an important opportunistic agent of pneumonia in the immunocompromised host. Currently, the diagnosis of Pneumocystis carinii pneumonia (PCP) is dependent on the morphological identification of Pneumocystis carinii organisms in bronchoalveolar lavage (BAL) or induced sputum (IS) specimens by histochemical or immunofluorescence staining. In a recent study we performed using polymerase chain reaction (PCR) technology applied to IS samples [1], we recorded increased diagnostic sensitivity in specimens with a small parasite load, as has been reported by others [2]. Less invasive than BAL, IS specimens have proved to be a useful means of detecting PCP; however, this is true only in laboratories that carefully control sputum induction and routinely evaluate these types of specimens using microscopy. Recently, the analysis of oral washes (OWs), a noninvasive and simple alternative to IS, using PCR assays has shown good sensitivity in the diagnosis of PCP, but their usefulness has not been studied in cases of PCP with a low parasite burden and in cases of subclinical infection.

This study was designed to assess the efficacy of using OWs analysed by conventional stain and Pneumocystis carinii (PC) nested PCR, performed after a simple DNA extraction procedure with proteinase K-boiling, to diagnose cases of PCP with a low parasite burden and cases of asymptomatic Pneumocystis carinii carriage.

Materials and Methods

A total of 104 paired IS and OW specimens were collected from 104 HIV-seropositive patients with respiratory symptoms between April 1997 and November 1998 at the Department of Infectious Diseases, Santa Maria Hospital, Lisbon, Portugal and evaluated for Pneumocystis carinii. OWs were collected before sputum induction by the patients rinsing their oral cavities with 10 ml sterile saline and gargling for about 1 min. As control samples, 32 OWs were obtained from 32 immunocompetent healthy individuals who did not agree to be submitted to the sputum induction procedure.

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Effect of Oral Washes on the Diagnosis of Pneumocystis carinii Pneumonia with a Low Parasite Burden and on Detection of Organisms in Subclinical Infections

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All samples were treated with an equal volume of 0.3% dithiothreitol (DTT; Sigma, Canada) for 30 min at 37°C, and cells were centrifuged at 2,500g for 10 min. The supernatant was discarded and the cell pellet was resuspended with 0.5 ml of phosphate-buffered saline (PBS; bioMérieux, France); one aliquot was stored at –70°C for later analysis by PCR. IS and OW specimens were analysed by direct immunofluorescence with monoclonal antibodies specific for Pneumocystis carinii (Cellabs, Australia), according to the manufacturer’s instructions, and by methenamine silver stain and PC-mtrRNA nested PCR [1]. Microbiologists performing the PCR technique were blinded to the results of conventional stains and to the patients’ clinical data. Semiquantitation of Pneumocystis carinii cysts, identified by conventional staining of the IS samples, was done by personnel experienced in the routine use of microscopy to diagnose PCP. The quantity of cysts was determined as follows: few (1–3 cysts in 30 fields at ×1,000), moderate (4–30 cysts in 30 fields at ×1,000) and heavy (2 or more cysts in one field at ×1,000).

A case of PCP was defined as the visualisation of Pneumocystis carinii organisms in IS specimens or the detection of Pneumocystis carinii DNA in IS specimens by nested PCR in patients with a clinical diagnosis of PCP, defined by the presence of at least two of the following variables: symptoms such as unproductive cough, fever and dyspnoea, suggestive chest radiographs, PaO2 <65 mm Hg and positive response to anti-PCP treatment. A case of PCP with a low parasite load was considered to be a case in which few organisms were identified by conventional stains or a case in which Pneumocystis carinii DNA was detected in the IS specimen of a patient with a clinical diagnosis of PCP. A subclinical infection was defined as a case in which few parasites were identified in the IS specimen or a case in which Pneumocystis carinii DNA was detected in a specimen, without being supported by a clinical diagnosis of PCP. Those patients who did not meet these criteria were considered Pneumocystis carinii negative.

After treatment with DTT, a 100-µl volume of pulmonary specimen was digested with proteinase K at 56°C for 2 h, followed by incubation at 95°C for 10 min. Two microliters of the digested pulmonary specimen was used directly for PCR [3].

PCR was performed on a specimen with the outer primer pair pAZ 102-E and pAZ 102-H for the first amplification round and the internal primer pair pAZ 102-X and pAZ 102-Y for the second round to amplify a 263-bp sequence [4, 5]. Samples that resulted negative using this PCR procedure were amplified with a primer pair (BG1 and BG2) specific for the human β-globin gene [6] to exclude inhibition of PCR. The PCR products were electrophoresed on a 3% agarose gel and visualised using ethidium bromide staining. Standard procedures were used to prevent the contamination of specimens. DNA extraction, amplification and product detection procedures were carried out in separate areas of the laboratory. Negative controls (i.e., all the reaction mixture components minus the DNA template) and positive controls (i.e., a suspension of Pneumocystis carinii DNA) were run with each experiment.

### Table 1 Detection of Pneumocystis carinii according to the type of infection and parasite load in 104 paired, induced sputum (IS) and oral wash (OW) specimens using conventional stain and PC-mtrRNA nested PCR

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>No. (%) of IS specimens</th>
<th>No. (%) of OWs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with PCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate or high parasite load</td>
<td>24/52 (46)</td>
<td>15/24 (63)</td>
</tr>
<tr>
<td>Low parasite load</td>
<td>28/52 (54)</td>
<td>11/28 (39)</td>
</tr>
<tr>
<td>Subclinical P. carinii infection</td>
<td>15/104 (14)</td>
<td>4/15 (27)</td>
</tr>
<tr>
<td>No P. carinii infection</td>
<td>37/104 (36)</td>
<td>74/104 (71)</td>
</tr>
</tbody>
</table>

a Defined in Materials and Methods

**Results and Discussion**

A total of 104 paired IS and OW specimens obtained from 104 HIV-seropositive patients were evaluated for Pneumocystis carinii, as were 32 OWs collected from 32 immunocompetent healthy individuals, which were used as controls. The 32 control OWs were all negative by conventional stain and PCR. Table 1 summarises the results obtained using the samples from the 104 HIV-seropositive patients. Fifty-two Pneumocystis carinii-positive IS specimens were obtained from patients with PCP. Of these 52 PCP cases, 26 were detected in the OWs by conventional stain or by PCR. Twenty-four of the PCP cases were determined to have a high or moderate parasite load, and 15 of them had OWs positive by conventional stain or PCR. Twenty-eight of the PCP cases had a low parasite load, and the parasite was detected in 11 of the corresponding OWs. Fifteen of the 104 IS specimens studied belonged to those patients with subclinical infection or asymptomatic carriage. Using conventional stain or PCR, we were able to detect four of these cases in the OWs. Pneumocystis carinii was not detected in 37 IS specimens and in 74 OWs.

Fourteen of the 104 IS samples and 18 of the 104 OWs studied resulted negative using PC-mtrRNA nested PCR and β-globin PCR, but 4 of 14 IS samples and 10 of 18 OWs resulted positive using microscopy. Several studies have examined the efficacy of using noninvasive measures to diagnose PCP (i.e., OW specimens examined by PCR) in immunocompromised patients with acute respiratory symptoms; all of them included patients in whom PCP had been verified clinically and microscopically, usually with a moderate parasite load, using pulmonary specimens [7, 8, 9, 10]. Recent studies have found that PCR detection of PCP organisms in BAL and IS specimens obtained from HIV-seropositive and -seronegative patients without PCP, suggest cases of subclinical infection [1, 11, 12, 13, 14]. In a previous study, we also detected PCP in patients with a low parasite burden of Pneumocystis carinii lung infection [1]. The role played by these types of infection, especially asymptomatic carriage, in the transmission of this organism is still being established. To properly evaluate the use of OWs in PCP diagnosis, we therefore collected paired OW and IS specimens from patients with respiratory symptoms whose samples were going to be examined in a laboratory for the presence of Pneumocystis carinii and OWs from immunocompetent healthy individuals, which were used as controls. By using conventional stain and PC-mtrRNA nested PCR on these OWs and comparing the results with those obtained using the paired IS specimens, we intended to determine if such simple noninvasive biological specimens could be used to detect cases of PCP with a low parasite load and cases of subclinical infection.