Allele-Specific PCR Method Based on \textit{pncA} and \textit{oxyR} Sequences for Distinguishing \textit{Mycobacterium bovis} from \textit{Mycobacterium tuberculosis}: Intraspecific \textit{M. bovis} \textit{pncA} Sequence Polymorphism

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An allele-specific amplification method based on two genetic polymorphisms to differentiate \textit{Mycobacterium tuberculosis} from \textit{Mycobacterium bovis} was tested. Based on the differences found at position 169 in the \textit{pncA} genes from \textit{M. tuberculosis} and \textit{M. bovis}, a PCR system which was able to differentiate most of the 237 \textit{M. tuberculosis} complex isolates tested in one of the two species was developed. All 121 \textit{M. tuberculosis} strains showed the expected base (cytosine) at position 169. Most of the \textit{M. bovis} isolates had a guanine at the cited position. Nevertheless, 18 of the 116 \textit{M. bovis} isolates, all of them goat isolates, showed the \textit{pncA} polymorphism specific to \textit{M. bovis}. These results suggest that \textit{M. bovis} may be the nicotinamidase-missing link at the origin of the \textit{M. tuberculosis} species. Based on the polymorphism found at position 285 in the \textit{oxyR} gene, the same system was used to differentiate \textit{M. tuberculosis} from \textit{M. bovis}. In this case, DNAs from all 121 \textit{M. tuberculosis} isolates had the expected base (guanine) at this position. In addition, all 116 \textit{M. bovis} isolates, including those from goats, showed the identical polymorphism (adenine). The \textit{oxyR} allele-specific amplification method can differentiate \textit{M. bovis} from \textit{M. tuberculosis}, is rapid (results can be obtained in less than 3 h), and is easy to perform.

While most cases of human tuberculosis are caused by \textit{Mycobacterium tuberculosis}, infections caused by \textit{Mycobacterium bovis} are being increasingly documented, in some cases in epidemic nosocomial bursts (2, 3, 8). Classical methods to differentiate both species are based on nitrate reduction, pyrazinamidase activity, pyrazinamide susceptibility, niacin accumulation, and growth in thiophene-2-carboxylic acid hydrazide-containing media. Some of these assays are tedious and are infrequently performed by diagnostic laboratories. However, from the clinical and epidemiological perspective, a rapid method to differentiate \textit{M. tuberculosis} from \textit{M. bovis} is urgently needed, both for treatment (due to pyrazinamide and even-
method. In this case, a 548-bp fragment of oxyR is amplified. Agarose gel electrophoresis is performed to verify the amplification of the desired fragment before Alul digestion. The result of the restriction is visualized by a new electrophoresis.

In the last few years, several methods that detect single nucleotide changes in DNA fragments have been described (for a review, see reference 6). The allele-specific PCR method was originally described in 1989 (14) and later improved (7, 13). In this report, we describe the use of an allele-specific PCR method to detect the polymorphisms in the pncA and oxyR genes with the objective of rapidly and easily differentiating M. bovis from M. tuberculosis. The first system we tested was based on the nucleotide polymorphism at position 169 in the pncA gene. This system failed to differentiate M. bovis isolated from goats from M. tuberculosis strains. To overcome this limitation, we assayed another polymorphism, the guanine or adenine at position 285 in the oxyR gene. The diagnostic possibilities of both systems were evaluated with 237 strains belonging to the M. tuberculosis complex. (This work was presented in part at the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada, 28 September to 1 October 1997.)

**MATERIALS AND METHODS**

Strains and DNA. The M. tuberculosis strains used in this study were either isolated from different patients from the Hospital Infantil de México Federico Gómez, México D.F., México (80 strains), or the Hospital Ramón y Cajal, Madrid, Spain (31 strains), or were from the Universidad de Zaragoza collection (10 strains). The M. bovis isolates were from the Facultad de Medicina, Universidad de Zaragoza (105 strains), the Facultad de Veterinaria of the Universidad Complutense de Madrid (10 strains), and the Hospital Ramón y Cajal (1 strain). The M. tuberculosis strains were isolated from human patients. The M. bovis strains had different origins, including humans, cattle, and goats. Chromosomal DNA was purified as previously described (16).

Species characterization. M. tuberculosis complex determination was made by classical methods (12) and by the PCR restriction enzyme pattern analysis method previously described (24). This method is based on the different restriction enzyme patterns of a PCR-amplified DNA fragment in the gene encoding the 65-kDa mycobacterial protein. Previously described biochemical tests were used to distinguish M. bovis from M. tuberculosis (10, 12, 15).

Primers and PCR conditions. The amplification primers for pncA and oxyR used in this study were based on previously described sequences (5, 20). For the allele-specific pncA method, the forward primer, pncATB-1.2 (5′-ATGCGGC GGTGATCATCGTC-3′), complements bases 1 to 21 in the gene. The reverse primers, pncAMT-2 (5′-CGGTTGCGGAGAAGCGG-3′) and pncAMB-2 (5′-CGGTGCGGAGAAGCGG-3′), complement bases 185 to 168. For the oxyR allele-specific method, the forward primer was oxyRTB-2.1 (5′-TGGCCGG GGTCTCGGCGG-3′) and the reverse primers were oxyRMT-1 (5′-GACACG ACCTGTGGCGAAGCGG-3′) and oxyRMB-1 (5′-TGACAGCACTGGCCAGG GTAA-3′). (The underlined bases are changes included in the primer sequences and do not complement the published sequences. The boldfaced bases complement those present in the M. tuberculosis or M. bovis sequences.) For each allele-specific method, DNA samples were subjected to two differential amplifications in two separate tubes. Both reactions were performed with the same forward primer, pncATB-1.2 or oxyRTB-2.1, and one of the two discriminator primers, pncAMT-2 or oxyRMT-1 for M. tuberculosis or pncAMB-2 or oxyRMB-1 for M. bovis. The M. tuberculosis-specific amplification reaction mixture contained 1 μl of template DNA (about 100 ng) and 24 μl of a solution consisting of the following: 200 μM each deoxynucleoside triphosphate, 25 pmol of each primer (pncATB-1.2 and pncAMT-2 for pncA or oxyRTB-2.1 and oxyRMT-1 for oxyR), 1.0 U of Taq-Gold DNA polymerase (Perkin-Elmer), 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 2 mM MgCl2, and 0.01% gelatin. The M. bovis-specific amplification reaction mixtures were identical to those of M. tuberculosis but contained primer pncAMB-2 instead of pncAMT-2, for pncA, and oxyRMB-1 instead of oxyRMT-1, for oxyR. For the pncA method, the cycling parameters were 95°C for 12 min, followed by 30 three-step cycles including denaturation at 94°C for 1 min, annealing at 67°C for 1 min, and extension at 72°C for 1 min. For the oxyR method, the cycling parameters were 95°C for 12 min, followed by 30 two-step cycles including denaturation at 94°C for 45 s and annealing plus extension at 70°C for 1 min 30 s. The amplification products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide fluorescence. A unique amplification product of 185 bp (pncA) or 270 bp (oxyR) must be visualized in either M. tuberculosis or M. bovis reactions.

**RESULTS**

Design of allele-specific primers and optimization of PCR conditions. A common forward primer, pncATB-1.2, and two reverse allele-specific primers, pncAMT-2 and pncAMB-2, were designed to hybridize with the published sequences of M. tuberculosis and M. bovis pncA genes. The reverse primer pncAMT-2, which was designed to anneal only to the pncA sequence from M. tuberculosis and not to that from M. bovis, has a mismatch in the third nucleotide from the 3′ end (corresponding to position 170 in the gene). It is expected that this mismatch will prevent amplification only when the template sequence also contains an additional change. The M. tuberculosis pncA gene complements pncAMT-2 except for the mismatch, while the M. bovis gene, which has a change from C to G at position 169, shows two mismatches, one at position 170 and the other at position 169, corresponding to the polymorphism that differentiates M. tuberculosis from M. bovis. In contrast, the amplification of M. bovis pncA, but not that of M. tuberculosis, will occur when the reverse primer for M. bovis, pncAMB-2, is used. Figure 1 shows the principle of the method.

One hundred nanograms of genomic DNA from M. tuberculosis H37Rv and the same amount of DNA from a human isolate of M. bovis were subjected to amplifications with the cited primers. After several rounds of amplifications, testing different annealing temperatures and MgCl2 concentrations (data not shown), adequate conditions (see Materials and Methods) to distinguish the two alleles with the complementary reactions were found. The oxyR allele-specific PCR system was developed in a similar way.

**FIG. 1.** Diagram showing the principle of the allele-specific PCR method to detect the polymorphism at position 169 in the pncA gene. pncATB-1.2 is a forward primer able to hybridize both M. tuberculosis and M. bovis pncA genes; pncAMT-2 is a reverse primer presenting a single mismatch with the M. tuberculosis pncA gene and two mismatches with the M. bovis gene; pncAMB-2 is a reverse primer presenting two mismatches with the M. tuberculosis pncA gene and a single mismatch with the M. bovis gene. Only single mismatches allow PCR amplification. The identical principle was applied to detect oxyR polymorphism.

**Rapid differentiation of M. bovis from M. tuberculosis by allele-specific PCR.** Different results were obtained with the pncA and oxyR systems.

(i) **pncA system.** The pncA allele-specific PCR system was applied to the control strain M. tuberculosis H37Rv (ATCC
specific amplification performed with DNAs from representative bovine and goat *M. bovis* and *M. tuberculosis* strains.

**Sensitivity of the technique.** To determine the sensitivity of the technique, both *M. tuberculosis* and *M. bovis* DNAs were serially diluted and subjected to amplification by the two cited systems. Amplicons of 185 or 274 bp were visualized when amplifications were performed with as little as 20 pg of chromosomal DNA. Both systems, *pncA* and *oxyR*, had similar sensitivities (data not shown).

**DISCUSSION**

DNA sequencing has shown that *pncA* (20) and *oxyR* (5) genes from *M. bovis* strains contain changes at positions 169 and 285, respectively, with respect to the *M. tuberculosis* sequence. Based on these polymorphisms, single-strand conformational polymorphism (18) and PCR-RFLP (21) assays were developed. Our allele-specific PCR tests applied the same principle as that of these previous assays but with the advantages of having a shorter response time (less than 3 h) and being simpler and safer (no acrylamide gels required) as well as inexpensive (minimal volume of reagents and no restriction enzymes). Our results with *oxyR* confirm those of Sreevatsan et al. (21, 22), who found that the polymorphic site located at nucleotide 285 uniformly distinguished *M. bovis* (adenine) from non-*M. bovis* isolates. Furthermore, our work shows that the allele-specific PCR based on the *oxyR* polymorphism at nucleotide 285 is a useful technique for distinguishing *M. bovis* from *M. tuberculosis*.

The study of polymorphism at nucleotide 169 in the *pncA* gene shows that not all *M. bovis* strains contain the same nucleotide (guanine) at this position. In a recent study, Sreevatsan et al. (23) found a cytosine instead of a guanine at position 169 in two *M. bovis* isolates. Interestingly, all 18 *M. bovis* strains in our study presenting this particular substitution were isolated from goats. The position-169 polymorphism test was also performed with a large number of *M. tuberculosis* strains (131 strains), including 21 pyrazinamide-resistant strains. In all cases, the absence of the C-to-G mutation at position 169 was documented. This observation also confirms those made by Sreevatsan et al. (23) and Scorpio et al. (19), who did not find the *M. bovis* *pncA*-specific mutation in pyrazinamide-resistant *M. tuberculosis* strains.

By introducing the functional *M. tuberculosis pncA* gene into *M. bovis* BCG strains, Scorpio and Zhang (20) restored the pyrazinamide sensitivity of these strains. With this experiment, they demonstrated that the His-to-Asp change at position 57 in the *pncA* product is actually the change responsible for the lack of pyrazinamidase and nicotinamidase activities. The absence of this change among the almost 100 pyrazinamide-resistant isolates previously tested (21 from this study) (19, 23) shows that this mutation is not present, or very uncommon, among pyrazinamide-resistant *M. tuberculosis* strains. The explanations for the natural occurrence of a C-to-G mutation at position 169 in *M. bovis* remain unclear, considering that the same mutation appears to be unfavorable for pyrazinamide-resistant *M. tuberculosis*.

Organisms containing genes that encode active enzymes may evolve (by mutation and selection) increased enzyme activity in environments where the resulting phenotype provides a higher fitness. A decrease or loss of the activity may also be the result of a reduced fitness associated with exposure of the phenotype to another environment. *M. tuberculosis* has been suggested to have arisen from *M. bovis* (11), but that implies an ancestral *M. bovis* with nicotinamidase activity. Molecular epidemiology studies have recently shown that *M. bovis* strains isolated from

27294) and 100 *M. tuberculosis* strains of human origin (México D.F., Zaragoza, and Madrid, Spain). In all cases, a positive amplification was obtained with the *M. tuberculosis*-specific primer *pncAMT*-2 but not with the *M. bovis*-specific primer *pncAMB*-2.

In *M. tuberculosis* strains, the acquisition of mutations rendering the product of *pncA* inactive is considered to result in pyrazinamide resistance. The change at position 169 of the gene from *M. bovis* is responsible for the lack of pyrazinamidase activity (which explains its natural resistance to the compound). The existence of pyrazinamide-resistant *M. tuberculosis* strains having this mutation cannot be ruled out. To test this possibility, we performed the *pncA* allele-specific amplification reactions with DNA extracted from 21 pyrazinamide-resistant *M. tuberculosis* strains of different origins. In all cases, a positive amplification fragment of 185 bp was obtained with the *M. tuberculosis*-specific reverse primer but not with the *M. bovis* one. This indicates that none of these strains contains a mutation at position 169.

The same protocol was applied to 116 *M. bovis* strains of animal origin (Zaragoza and Madrid) and to 1 *M. bovis* strain of human origin (Madrid). With 99 of 117 (85%) of these strains, a positive amplification was obtained with *pncAMB*-2, and *M. bovis*-specific primer, but not with *pncAMT*-2. The existence of 18 strains of *M. bovis* not containing the expected *M. bovis pncA* polymorphism casts doubt on the validity of this system for use as a discriminating technique. Interestingly, the 18 strains were, in all cases, isolated from goats. Figure 2 shows the results of the *pncA* allele-specific amplification performed with DNAs from representative strains of bovine *M. bovis*, goat *M. bovis*, and *M. tuberculosis*. The entire nucleic acid sequences of the *pncA* genes from six *M. bovis* strains (three isolated from goats, two from cattle, and one from a human) were obtained. *pncA* genes from three *M. tuberculosis* strains were also sequenced. The sequences obtained fit the described polymorphisms (data not shown).

(ii) *oxyR* system. In contrast to the results with the *pncA* system, all of the *M. bovis* strains tested (including those isolated from goats) had the described *M. bovis*-specific polymorphism (an adenine residue at position 285) in the *oxyR* gene. In addition, all *M. tuberculosis* strains had a guanine residue at the cited position. Figure 2 shows the results of the *oxyR* allele-specific amplification performed with DNAs from representative bovine and goat *M. bovis* and *M. tuberculosis* strains.
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REFERENCES

AUTHORS’ CORRECTION
(2 articles)

Genetic Characterization of Multidrug-Resistant Mycobacterium bovis Strains from a Hospital Outbreak Involving Human Immunodeficiency Virus-Positive Patients

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Allele-Specific PCR Method Based on pncA and oxyR Sequences for Distinguishing Mycobacterium bovis from Mycobacterium tuberculosis: Intraspecific M. bovis pncA Sequence Polymorphism

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The following correction pertains to both of the above articles.
The Acknowledgments section should have indicated that Luz Elena Espinosa de los Monteros was a student from Programa Multidisciplinario de Biomedicina Molecular from CINVESTAV-IPN and the recipient of a fellowship from CONACYT.