Polymerase Chain Reaction (PCR) Technique in Detection of Actinomyces SPP by Using Cervico-Vaginal Fluid Samples

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OBJECTIVE: To detect the most common Actinomyces species correctly in cervico-vaginal fluid samples by using Polymerase Chain Reaction (PCR) technique.

STUDY DESIGN: Cervico-vaginal fluid samples were obtained from 200 patients and bacterial genomic DNA was isolated directly from samples of each patient. The PCR technique was used for two purposes. First was applied for the detection of Actinomycetales including the genus Actinomyces and second was used for the differentiation of Actinomyces spp from each other.

RESULTS: Actinomycetales was detected in 16 (8%) of 200 samples by PCR. In these 16 examined samples, 8 were positive for at least one of the following three species, namely A. meyeri (2.5%), A. viscosus (1%) and A. israelii (0.5%). No A. odontolyticus was detected in any of the fluid samples.

CONCLUSION: PCR was shown to be a simple, rapid and highly discriminatory method for the identification and differentiation of Actinomyces spp in examining cervico-vaginal fluid samples.

Key Words: Actinomyces, Polymerase chain reaction (PCR), Cervico-vaginal fluid samples

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Introduction

The genus Actinomyces comprises a group of anaerobic, non-spore forming, non-motile, non-acid-fast and Gram-positive bacteria that have a G+C content of 55 to 71 mol%.[1-5] The natural habitats of many Actinomyces spp are the mucosal surfaces of oral, gastrointestinal and genital tracts[6] and they do not penetrate the intact mucosa under normal conditions. However, a foreign body, neoplasia and Diabetes mellitus can be predisposing factors for disrupting the mucosa. When mucosal injury has occured, these oppurtunistic organisms can be pathogenic and cause classical actinomycosis. Actinomyces spp. grows grossly and slowly by breaching anatomical boundaries and forms abscesses and sinus tracts filled with many neutrophils and surrounded by dense fibrotic tissue.[6-8] Lesions become firm as they enlarge and may form fistulas between organs or to the skin.[7]

Identification of genital Actinomyces spp is generally challenging and unreliable.[5] Among diagnostic techniques for identifying genital Actinomyces, Pap test lacks specificity and sensitivity.[9] Although highly suggestive of actinomycosis, histopathological examination of sections of tissue alone do not confirm the diagnosis. Culture is considered as gold standart for identifying this organism, but Actinomyces spp. are fastidious, slow-growing bacteria and sensitive to most antibiotics and these characteristics make the organism difficult to identify using routine specimen handling and routine culture techniques.[9,10] In contrast to these techniques, in recent years molecular techniques including PCR and 16S rRNA gene sequencing have resulted in much improved identification and differentiation of closely related species.[11,12] Taken together these data, it is plausible to indicate that the actual sensitivity of other techniques such as Pap stain detection and microbiological evaluation is unsatisfactory. Therefore, the aim of this study was to employ molecular methods, including direct DNA extraction from clinical samples, to identify and differentiate Actinomyces species in cervico-vaginal fluid samples.

Material and Method

Study population

A total of 200 women aged 19-78 years with varied gynecologic complaints were seen in the outpatient clinic in the Department of Gynecology and Obstetrics, Hacettepe

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University, Ankara, Turkey. Before pelvic examination, a questionnaire that requested information on age, menstruation date, gravidity and clinical symptoms were completed for all women enrolled in this study. Pregnant women were not included.

Clinical sampling and direct genomic DNA isolation

Cervico-vaginal fluid samples were collected from 200 patients with a sterile swab. Then, they were placed in tubes containing Tris-EDTA (TE) buffer and transported to the Cytology Laboratory, Department of Biology, Hacettepe University. Before DNA isolation, the tubes with swabs were vigorously vortexed for 30 seconds in order to ensure disaggregation of all bacteria into the TE buffer. Afterwards, the swabs were aseptically removed from the suspension and the bacterial suspension was pelleted by centrifugation for 3 min at 10.000 rpm. Afterwards, the pellet was resuspended in 400 µl lysis/binding solution of A101 DNA Extraction Kit. Subsequently, total bacterial genomic DNA was isolated, according to the protocol recommended by the manufacturer (Metis Biotechnology, Turkey). Confirmation of DNA (3 µl/sample) was determined by electrophoresis on a 0.8% agarose gel, viewed with an ultraviolet light box.

PCR Procedure

Extracted bacterial genomic DNAs were used as a template for two seperate PCR. First PCR was set up to amplify 16S rRNA genes of Actinomycetales and second PCR was set up to amplify 16S rRNA genes of four Actinomyces species using appropriate primers (Table I).

PCR Primers

The universal primers for the order of Actinomycetales, which includes the genus Actinomyces, were used as published.[12] Four Actinomyces species-specific primers were designed from the 16S rRNA genes of these species. Firstly, the sequences of the 16S rRNA subunits for Actinomyces israelii (Accession M33912), Actinomyces viscosus (Accession X82453), Actinomyces naeslundii (Accession M33911), Actinomyces meyeri (Accession X82451) and Actinomyces odontolyticus (Accession M33910) were downloaded from GenBank, National Center for Biotechnology Information and aligned using BioEdit Sequence Alignment Programme. Variable regions of the highly homologous sequences were used to synthesize a forward species-specific primer for each of the four species tested. Same reverse primer was used for these organisms. For a pair of universal primers for the order of Actinomycetales and species-specific primer sequences, see Table I. The specificity of the primers was also tested by a BLAST search (National Center for Biotechnology Information).

PCR Amplification

PCR reactions containing primer pairs for the order of Actinomycetales, including Actinomyces genus, were run for each 200 clinical samples. Seperate PCR reactions containing species-specific primer pairs were run for only Actinomycetales-positive samples for speciation of Actinomyces spp. Each PCR reaction consisted of 10X PCR Buffer, 4 µl MgCl2, 0.5 µl dNTP, 10 µl template. The PCR reaction was conducted in a thermal cycler under the flollowing parameters: initial denaturation for 1 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1,5 minute at 72 °C. After the main cycle program was complete, an additional extension step was added for 10 minutes at 72 °C. The PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide, viewed on an ultraviolet light box and photographed. Amplicon size was analyzed by comparison to a ladder (ΦX174 DNA-HaeIII Digest). PCR positive controls were used with DSMZ type strain DNA and negative controls with distilled water.

Table 1: PCR primers used in this study

DNA Template	Primer pairs (5' ⟶ 3')	Base position (amplicon lengths, bp)
Actinomyces meyeri	5'-TCTGCGATTACTAGCGACTCC-3' 5'-CCACCCGTGGTTTTCTGCG-3'	818-1337 (519)
Actinomyces viscosus	5'-TCTGCGATTACTAGCGACTCC-3' 5'-TCGTAGGCGGCTGGTCGC-3'	538-1323 (785)
Actinomyces odontolyticus	5'-TCTGCGATTACTAGCGACTCC-3' 5'-CGGCACTGCAGAGATGTYGTGG-3'	888-1241 (353)
Actinomyces israelii	5'-TCTGCGATTACTAGCGACTCC-3' 5'- GGGCCTCCCCTTTTTGGGCC-3'	783-1122 (339)
Actinomycetales	5'-GGCKTGCGGTGGTACGGGC-3' 5-GGCTTTAAGGGATTCGCTCCRCCTCAC-3'	632-1306 (675)

Results

The specificity of each primer designed to amplify 16S rRNA gene of A. israelii, A. meyeri, A. viscosus and A. odontolyticus was tested against several closest species from the same genus. No cross-reactivity was observed (Figure 1). Positive controls with the type strain yielded the expected size amplicon for each organism tested. There were no bands observed in negative controls.

DNA extracted from a total of 200 cervico-vaginal samples and 16 clinical samples reacted positively with the pair of primers we used for the PCR assay suggesting the presence of Actinomycetales in all 200 cervico-vaginal samples examined (Figure 2). When the PCR reactions with the species-specific primers were evaluated for these 16 examined samples, 8 were positive for at least one of the following three species, namely A. meyeri (2.5%) (Figure 3), A. viscosus (1%) and A. israelii (0.5%). A. odontolyticus was not detected in any of the cervico-vaginal fluid samples. The detection rate of A. meyeri was higher than the other two species.

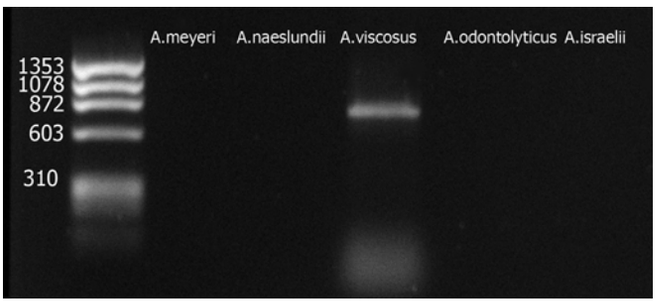

Figure 1: Specificity of PCR for the detection of A. viscosus. Lane 1: DNA ladder, Lane 4: contained positive control DNA (ATCC 15987) of A. viscosus. Lane 2,3,5,6: contained DNA of A. meyeri (ATCC 35568), A. naeslundii (ATCC 12104), A. odotontolyticus (ATCC 17982) and A. israelii.

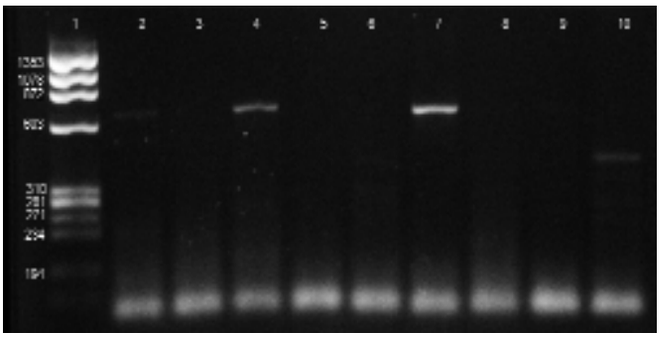

Figure 2: PCR survey of clinical samples. Lane 1: DNA ladder, Lane 2-9 were amplified using the universal primers for the order of Actinomycetales. Lane 4 and 7 demostrated the presence of Actinomycetales.

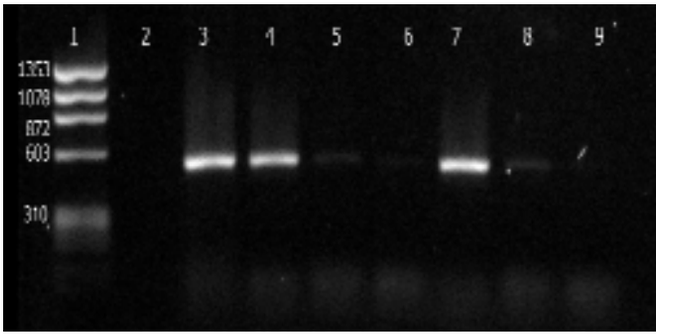

Figure 3: PCR survey of clinical samples reacted positively with the pair of universal primers for the order of Actinomycetales. Lane 1: DNA ladder, Lane 2-9 were amplified using A. meyeri-specific primers. Lane 2 contained distilled water as negative controls. Lane 3 contained positive control DNA of A. meyeri. Lane 4 and 7 demonstrated the presence of A. meyeri.

Discussion

Pelvic actinomycosis is frequently undiagnosed or misdiagnosed due to the number of other fungal infections which may present with similar manifestations. In most cases, definitive diagnosis is made after surgical resection.[13] Although there are varied diagnostic approaches, its preoperative diagnosis rate is stil lower than 10%.[14] The identification and differentiation of these anaerobic bacteria in clinical specimens is very difficult becuse of their complex transport and growth requirements and their fastidious and slow-growing nature.[15] Thus, in recent years, the improvement of molecular techniques such as PCR and 16S rRNA gene sequencing has provided much improved identification and differentiation of closely related Actinomyces species.[11,12] This study is sought to determine if PCR can be used to detect several of Actinomyces species associated with pelvic actinomycosis using 16S rRNA gene-specific primers (Table I) and in 8 (4%) of 200 examined samples were positive for at least one of three Actinomyces species. These species were A. meyeri, A. viscosus and A. israelii. These results show that PCR targetting 16S rRNA region can be used to detect Actinomyces spp. in genital tract samples correctly. This study also confirms that A. meyeri and A. viscosus can also be found in healty female genital tract.

A PCR method has several potential advantages in diagnosing of pelvic actinomycosis over the two most commonly used methods for detecting Actinomyces, the Pap test and culture. PCR is highly sensitive and can be optimized to pick up very low numbers of bacteria. This ability of PCR is very useful for identifying Actinomyces spp since this organism is present in low concentrations in healty and is not easily cultivated in disease because of its fastidious and slow-growing nature.[15] Another feature of PCR is that it quantifies DNA rather than viable organisms. This is also a potential advantage

because it can efficiently detect all Actinomyces, either dead or alive.[16] Like PCR, Pap test is not depend on viable organisms, but it is impossible to determine which Actinomyces spp is present with this test.[17] On the other hand, culture methods may not detect all bacteria since they are not viable and capable of growing into colonies. Thus, the diagnostic value of PCR is considered to be significant when Actinomyces spp are expected to be present in vaginal specimens.

In this study, we used methods to identify and differentiate Actinomyces spp from clinical samples directly. The methods included PCR with a pair of primers targeting the variable region of 16S rRNA gene of several Actinomyces species. The primers (Table I) did not used previously in our knowledge, and all have targeted the variable regions of 16S rRNA genes of four different Actinomyces species. Because, 16S rRNA gene is highly conserved within a species and among species of the same genus, it is considered the new standard for classification and identification of bacteria.[18,19] 16S rRNA sequences have been used to seperate several Actinomyes species from each other previosuly in literature. Our results are consistent with those of these studies.

In several previous studies, it is reported that A. israelii is the most common agent in pelvic actinomycosis.[6,9] In contrast to these studies, the detection rates of A.meyeri (2.5%) and A. viscosus (1%) were found higher than those of A. israelii (0.5%) in the present study. Furthermore, A. odontolyticus was not detected in any of the examined samples. According to these results, it is plausible to indicate that A.meyeri and A. viscosus may be isolated from genital tract of healty women, but not A. odontolyticus.

In conclusion, detection of Actinomyces by PCR analysis appears to be sensitive and specific. Advantages of this simple and rapid method compared to culture is the ability to detect low concentrations of organisms and to detect all Actinomyces, either dead or alive. Application of this method to further clinical samples may confirm its usefulness.

Actinomyces Türlerinin Serviko-Vajinal Sıvı Örneklerinde Polimeraz Zincir Reaksiyonu (PCR) Yöntemiyle Saptanması

AMAÇ: Serviko-vajinal sıvı örneklerinde, Polimeraz Zincir Reaksiyonu (PCR) tekniği kullanılarak en yaygın Actinomyces türlerinin saptanması.

GEREÇ VE YÖNTEM: Bu çalışmada 200 hastadan serviko-vajinal sıvı örneği alınmış ve her hastadan DNA izole edilmiştir. PCR yöntemi, iki amaç için kullanılmıştır. Bu amaçlardan birincisi, Actinomyces cinsinin dahil olduğu Actinomycetales'in tanımlanması, ikincisi ise örneklerde bulunan Actinomyces türlerinin tiplendirilmesidir.

BULGULAR: Actinomycetales, 200 örneğin 16'sında (%8) PCR yöntemi ile tespit edilmiştir. Bu 16 örneğin ise 8'inde, A. meyeri (%25), A. viscosus (%1) ve A. israelii (%0,5) türlerinden en az birinin olduğu belirlenmiştir. A. odontolyticus türü hiçbir sıvı örneğinde tespit edilmemiştir.

SONUÇ: PCR yönteminin, serviko-vajinal sıvı örneklerinden Actinomyces türlerinin tespit edilmesinde ve tiplendirilmesinde, basit, hızlı ve oldukça ayırt edici bir yöntem olduğu görülmüştür.

Anahtar Kelimeler: Actinomyces, Polimeraz zincir reaksiyonu (PCR), Serviko-vajinal sıvı örnekleri

References

1. Hall V, Talbot PR, Stubbs SL, Duerden BI. Identification of clinical isolates of Actinomyces species by amplified 16S ribosomal DNA restriction analysis. J Clin Microbiol 2001;39:3555-62.
2. Tang G, Samaranayake LP, Yip HK. Genotypic diversity of oral Actinomyces naeslundii genospecies 1 and 2 in caries-active preschool children. Oral Microbiol Immunol 2004;19:371-8.
3. Kaszuba M, Tomaszewska R, Pitynski K, Grzanka P, Bazan-Socha S, Musial J. Actinomycosis mimicking advanced cancer. Pol Arch Med Wewn 2008;118:581-4.
4. Joshi C, Sharma R, Mohsin Z. Pelvic actinomycosis: a rare entity presenting as tubo-ovarian abscess. Arch Gynecol Obstet 2009;281:305-6.
5. Fu PK and Tsai CA. Management of Patients with Huge Pelvic Actinomycosis complicated with Hydronephrosis: A Case Report. J Microbiol Immunol Infect 2010;43:442-6.
6. Carillo M, Valdez B, Vargas L, Alvarez L, Schorr M, Zlatev R, Stoytcheva M. In vitro Actinomyces israelii biofilm development on IUD copper surfaces. Contraception 2010;81:261-4.
7. Fiorino AS. Intrauterine Contraceptive Device-Associated Actinomycotic Abscess and Actinomyces Detection on Cervical Smear. Obstet Gynecol 1996;87:142-9.
8. Fitzhugh VA and Heller DS. Significance of a diagnosis of microorganisms on Pap smear. J Low Genit Tract Dis 2008;12:40-51.
9. Westhoff C. IUDs and colonization or infection with Actinomyces. Contraception 2007;75:48-50.
10. Wang YH, Tsai HC, Lee SS, Mai MH, Wann SR, Chen YS, Liu YC. Clinical manifestations of actinomycosis in Southern Taiwan. J Microbiol Immunol Infect 2007;40: 487-92.
11. Collins MD, Hoyles L, Kalfas S, Sundquist G, Monsen T, Nikolaitchouk N, Falsen E. Characterization of Actinomyces isolates from infected root canals of teeth: description of Actinomyces radicidentis sp. nov. J Clin Microbiol 2000;38:3399-403.

12. Xia T and Baumgartner JC. Occurrence of Actinomyces in infections of endodontic origin. J Endod 2003;29:549-52.
13. Ertan T. Kılıç M, Keşşaf Aşlar A. Yoldaş Ö, Göçmen E, Koç M. Actinomycosis of sigmoid colon: mimicking a colon cancer. The Journal of Selçuk Faculty of Medicine 2005;22:63-6.
14. Choi MH, Hong DG, Seong WJ, Lee YS, Park IS. Pelvic actinomycosis confirmed after surgery: single center experience. Arch Gynecol Obstet 2009;281:651-6.
15. Tamer A, Gündüz Y, Karabay O, İka H, Aksel F. Abdominal actinomycosis: a case report mimicking colon tumor. Türkiye Klinikleri J Med Sci 2006;26:330-3.
16. Schwebke JR and Lawing LF. Prevalence of Mobiluncus spp among women with and without bacterial vaginosis as detected by polymerase chain reaction. Sex Transm Dis 2001;28:195-6.
17. Kaya D, Demirezen Ş, Hasçelik G, Erciş S, Beksaç MS. The detection of genital Actinomyces by cytological and microbiological methods. Gynecol Obstet Reprod Med 2009;15:157-61.
18. Lau SKP, Woo PCY, Fung AMY, Chan K, Woo GKS, Yuen K. Anaerobic, non-sporulating, Gram-positive bacilli bacteraemia characterized by 16S rRNA gene sequencing. J Med Microbiol 2004;53:1247-53.
19. Elsayed S, George A, Zhang K. Intrauterine contraceptive device-associated pelvic actinomycosis caused by Actinomyces urogenitalis. Anaerobe 2006;12:67-70.