Seminars in
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Polymerase Chain Reaction-Based Molecular Diagnosis of Cutaneous Infections in Dermatopathology

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Conventional methods, including microscopy, culture, and serologic studies, are a mainstay in the diagnosis of cutaneous infection. However, owing to limitations associated with these techniques, such as low sensitivity for standard microscopy and in the case of culture delay in diagnosis, polymerase chain-reaction based molecular techniques have taken on an expanding role in the diagnosis of infectious processes in dermatopathology. In particular, these assays are a useful adjunct in the diagnosis of cutaneous tuberculosis, atypical mycobacterial infection, leprosy, Lyme disease, syphilis, rickettsioses, leishmaniasis, and some fungal and viral infections. Already in the case of tuberculosis and atypical mycobacterial infection, standardized polymerase chain-reaction assays are commonly used for diagnostic purposes. With time, additional molecular-based techniques will decrease in cost and gain increased standardization, thus delivering rapid diagnostic confirmation for many difficult-to-diagnose cutaneous infections from standard formalin-fixed paraffin-embedded tissue specimens.
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Conventional methods for the diagnosis of cutaneous infection in dermatopathology include those performed directly by the dermatopathologist, such as microscopy using histochemical stains and antigen detection using immunohistochemical (IHC) methods, as well as adjunctive laboratory techniques, including culture and serologic studies. These techniques are often adequate for the diagnosis of many infectious agents. However, each technique has inherent limitations. Conventional microscopy using special histochemical stains and IHC antigen detection is limited by low sensitivity if few organisms are present.[1] Culture isolation may require special media and long periods for growth; in addition, it may prove difficult to culture the particular microbe.[1,2] Serologic studies demonstrate variable sensitivity, depending on the stage of infection, and may require significant amounts of serum.[1] Fortunately, molecular techniques, such as polymerase chain reaction (PCR), can overcome many of these limitations. Because molecular techniques can detect small amounts or DNA or RNA, they can serve to quickly identify microorganisms that are present in small numbers in a clinical sample, stain poorly with conventional techniques, or are unculturable.[2] The application of PCR-based molecular techniques to the diagnosis of common infectious diseases encountered by the dermatopathologist is discussed.

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Tuberculosis

Although tuberculosis is often thought of as a disease of antiquity, it remains a significant global health problem, with a resurgence of infection worldwide.[3] Pulmonary tuberculosis is the most common presentation of disease, with extrapulmonary disease constituting 10% of all cases of tuberculosis, and cutaneous disease making up only a small number of those cases.[4] Cutaneous tuberculosis shows significant variability in its clinical presentation and is often difficult to distinguish from other granulomatous diseases with similar clinical and histopathologic features, such as cutaneous sarcoidosis and other cutaneous infections (eg, nontuberculous infections, deep fungal infection, and cutaneous leishmaniasis). Subsequently, the disease may not be suspected, and reliable laboratory tests are needed for a definitive diagnosis.

Intradermal testing using a purified protein derivative can be a helpful adjunct in diagnosis; however, it does not distinguish active from previous infections and may give nega-

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tive results in some forms of cutaneous tuberculosis.[5] Demonstration of acid-fast bacilli (AFB) using the Ziehl–Neelsen technique, combined with isolation of *Mycobacterium tuberculosis* on culture, remains the gold standard for the diagnosis of tuberculosis. It is important to keep in mind that the sensitivity of both techniques is often low in cutaneous disease, especially paucibacillary disease.[6] In addition, demonstration of AFB on Ziehl–Neelsen-stained sections does not distinguish tuberculosis from nontuberculous infections. Isolating the organism on culture only detects live organisms, and the identification of isolates may take up to 6 to 8 weeks using Lowenstein–Jensen medium.[4]

Molecular-based techniques are often superior to conventional techniques in the diagnosis of tuberculosis. There must be 5000-10,000 bacilli per milliliter of specimen to detect AFB on smears, and 10-100 live bacilli on culture.[7] In contrast, PCR can detect viable or nonviable mycobacteria from a sample containing < 10 organisms in 48 hours or less.[8] In addition, molecular-based techniques allow for the differentiation of *M tuberculosis* from atypical mycobacteria.[8]

Common molecular targets used include 16S rDNA and the insertion element IS6110.[9] Other regions that have been amplified include the *rpoB* gene encoding the β-subunit of the RNA polymerase, the 32-kDa protein gene, the *recA*, *hsp65*, *dnaj*, and *sodA* genes, and the 16S rRNA internal transcribed spacer.[9] The use of real-time PCR in the diagnosis of *M tuberculosis* infection is increasing because it has several benefits over conventional PCR, including reduced turnaround time owing to the combination of amplification and detection in 1 step, and improved sensitivity and specificity.[9,10]

Although the molecular tests for *M tuberculosis* described earlier in the text are most commonly used on respiratory sputum smears, these same techniques can be used on formalin-fixed paraffin-embedded (FFPE) tissue, including cutaneous specimens. Sensitivities for real-time PCR on FFPE tissue have ranged from 67% to 100%.[11] A disadvantage of using FFPE tissue is that the fixation procedure affects the quality of the DNA, resulting in potentially reduced sensitivity.[5,12] In addition, sensitivity may also be reduced in cutaneous tuberculosis owing to a paucity of organisms, especially in lupus vulgaris and tuberculosis verrucosa cutis.[12,13] The sensitivity of PCR in the diagnosis of cutaneous tuberculosis varies widely. One study evaluating 22 specimens, using primers specific for IS6110, detected *M tuberculosis* DNA in only 1 specimen (4.5%), which the authors attributed to a combination of degraded DNA, PCR-inhibiting substances, insufficient extraction of DNA, and inadequate sampling.[14] However, the majority of studies of cutaneous tuberculosis have demonstrated sensitivities ranging from 35% to 88%.[5,8,12,13] In most cases, the sensitivity of PCR is better than that of microscopic evaluation for AFB, and at least comparable or better than culture for the diagnosis of cutaneous tuberculosis.[8,12,13] PCR is a useful diagnostic adjunct for the dermatopathologist where rapid diagnosis is necessary in the setting of strongly suspected cutaneous tuberculosis with granulomatous inflammation but no demonstrable AFB.[15]

Atypical Mycobacteria

Atypical mycobacteria are opportunistic pathogens that can produce cutaneous infections as a result of direct inoculation associated with a surgical procedure or trauma as well as via hematogenous dissemination.[16] Similar to cutaneous *M tuberculosis* infection, nontuberculous mycobacterial infection demonstrates granulomatous inflammation (Fig. 1), and it is notoriously difficult to demonstrate microorganisms on Ziehl–Neelsen- or Fite-stained sections, with AFB being seen in only 13%-31% of cases (Fig. 2).[17,18] In addition, cultures may take several weeks to grow; in a case series of cutaneous *Mycobacterium marinum* infection, variable sensitivities ranging from as low as 3% to approximately 70%-80% were demonstrated.[17,19]

Similar molecular targets and commercially available molecular assays for *M tuberculosis* are used in the diagnosis of nontuberculous mycobacterial infection. 16S rDNA sequencing is the gold standard for the molecular diagnosis of nontuberculous infection.[20,21] However, 16S rDNA is limited in that it cannot discriminate between all the atypical mycobacterial species. In particular, infection with *M marinum* versus *Mycobacterium ulcerans* and *Mycobacterium abscessus* versus *Mycobacterium chelonae* cannot be differentiated using this method.[20,21] Instead, additional segments of 16S rDNA must be sequenced to differentiate these atypical mycobacteria.[20,21] Alternatively, other genes, including *hsp65*, the gene coding for the 32-kDa protein, and the 16S-23S rRNA internal transcribed spacer, allow for the differentiation of all clinically important atypical mycobacteria.[20]

Leprosy

Leprosy is a chronic infectious granulomatous disease secondary to the bacillus *Mycobacterium leprae* that preferentially affects the skin and peripheral nerves. Owing to its rarity in the United States, leprosy is a challenging diagnosis to make.[22] This difficulty is compounded by the fact that *M leprae* cannot be grown on synthetic culture media, and instead must be visualized using acid-fast staining techniques.

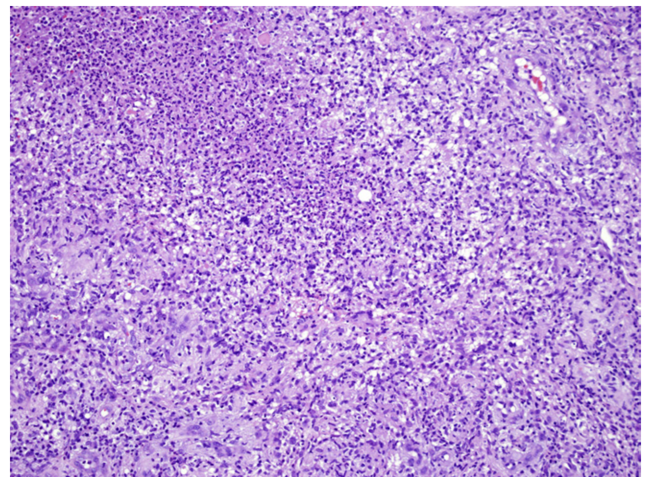

Figure 1 Nonspecific granulomatous and suppurative inflammation in the setting of cutaneous *M marinum* infection (hematoxylin and eosin, ×400).

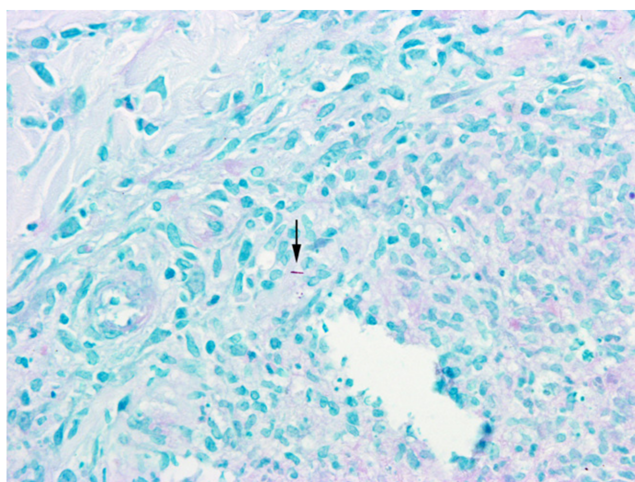

Figure 2 Focal beaded bacillus under black arrow in the setting of cutaneous *M marinum* infection, highlighting the difficulty in identifying organisms using standard histochemical staining in the setting of atypical mycobacterial infection in an immunocompetent host (Fite, ×600).

Visualizing the organism in skin-biopsy specimens or slit-skin smears is not difficult in multibacillary disease, with the slit smear giving positive results in nearly 100% of lepromatous and 75% of borderline leprosy cases.[22] In contrast, the organism is often problematic to identify in paucibacillary disease, with only 5% of slit smears demonstrating organisms in tuberculoid leprosy cases.[22]

Compared with histochemical staining techniques in which paucibacillary disease is difficult to diagnose, gene amplification can often detect the presence of *M leprae* from specimens with as few as 1-10 organisms.[23] These molecular tests include probes targeting both 16S rRNA and 16S rDNA as well as PCR of a 530-bp fragment encoding part of the 36-kDa protein gene. These techniques have proven useful in (1) evaluating specimens in which bacilli are sparse, (2) differentiating leprosy from other mycobacterial infections on specimens in which bacilli are numerous, but the clinical history is in question, and (3) evaluating response to therapy.[24,25] Sensitivities of these molecular assays have ranged from 34% to 80% in patients with paucibacillary disease to 100% in patients with multibacillary disease, with an overall specificity of 100%.[24-26]

Lyme Disease

Lyme disease is a multiorgan system disease caused by infection with the spirochete *Borrelia burgdorferi* and clinically manifests in 3 stages. The primary stage, erythema migrans, occurs in 50%-83% of Lyme disease cases and is associated with an expanding annular patch at the site of inoculation.[27] The diagnosis of early Lyme disease is usually based on the clinical features described earlier in the text and is often confirmed using serologic tests. However, in a patient with a history of living in or travelling to an endemic region, the dermatopathologist may be called on to aid in the diagnosis of erythema migrans from a biopsy specimen, especially in atypical clinical cases.

Serologic diagnosis in early disease is hampered by poor sensitivity; patients with a solitary lesion of erythema migrans are immunoglobulin M seropositive in 20%-40% of cases.[27-29] In addition, the histopathologic findings in erythema migrans are nonspecific and spirochetes are only demonstrable on silver-stained sections in 40% of cases.[30] The pathogen can be detected by culture; however, culture sensitivities range from 30% to 70% from skin biopsy specimens of erythema migrans and 5% from cerebrospinal fluid (CSF) in later-stage disease.[31]

PCR amplification of specific target genes of *B burgdorferi* sensu lato, including those coding for *flaB*, *recA*, *p66*, *ospA*, and some rRNA genes from skin, blood, CSF, and synovial fluid samples, have been used for diagnostic testing.[31,32] PCR sensitivity in the diagnosis of Lyme disease varies depending on the sample site, with skin demonstrating higher rates than blood and CSF samples.[33] Detection rates for *B burgdorferi* from skin biopsies of erythema migrans have ranged from 44% in FFPE specimens to as high as 90% in frozen tissue.[31] In addition, *B burgdorferi* DNA has also been detected in 54%-100% of cases of acrodermatitis atrophicans from Europe.[34] In erythema migrans, greater sensitivity is obtained from specimens obtained from the edge area of the lesion rather than from the center.[31] Specificity of PCR samples from all body sites is approximately 100% in all stages of disease.[32]

Syphilis

Treponema pallidum, the infectious agent of syphilis, can be problematic to detect. Unlike most bacteria, *T pallidum* cannot be isolated using the standard culture technique. In early syphilis, the diagnosis is often made by microscopic identification of the organism. A primary chancre, the cutaneous lesion of early congenital syphilis, and moist secondary-stage lesions (condyloma latum or mucous patches) containing large numbers of organisms can be diagnosed via dark-field examination or direct fluorescent antibody test for *T pallidum*.[35] However, a negative test result does not exclude the diagnosis because the lesion may be altered by healing or systemic/topical treatment, resulting in too few organisms being present for microscopic identification (sensitivity of

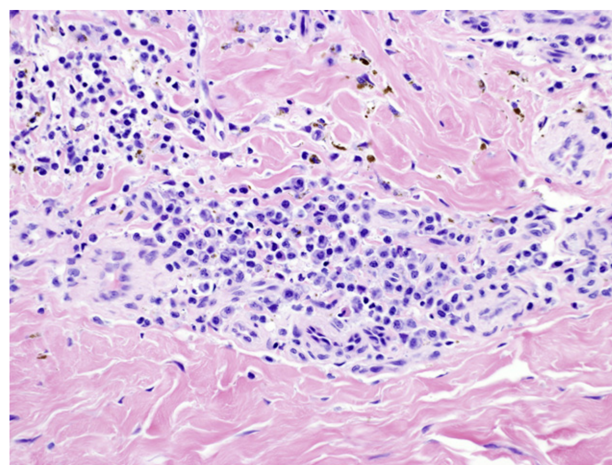

Figure 3 Nonspecific plasma cell–rich infiltrate in cutaneous biopsy of secondary-stage syphilis (H&E, ×400).

approximately 10^5 organisms/mL for dark-field examination).[35,36] Microscopic methods performed on FFPE biopsy specimens from cutaneous lesions, such as silver staining or immunohistochemistry, demonstrated a sensitivity of 25% for Dieterle staining and 48% for IHC detection of *T pallidum* in one study involving 1 case of primary, 26 cases of secondary, and 9 cases of tertiary syphilis to 41% for Steiner staining.[37] In another study of 17 cases of secondary syphilis, a 71% sensitivity for IHC detection of *T pallidum* was demonstrated.[38] However, such techniques are sometimes difficult to interpret owing to nonspecific background staining, especially using silver staining methods.[38] As the disease progresses in stage, the spirochete becomes scarce and challenging to identify (Figs. 3 and 4).[39] Serologic testing, a mainstay in the diagnosis of secondary and tertiary syphilis, is insensitive early in infection, plagued by lack of specificity, and cannot easily differentiate recent reinfection from past infection, thus making PCR an attractive testing adjunct.[35,40]

Several PCR methods and target genes for the diagnosis of *T pallidum* infection have been used, including the 47-kDa protein gene, *tpf-l* gene, basic membrane protein gene, and *tmpA* and *tmpB* genes.[1,41] The sensitivity of these assays varies from the equivalent of 10^{-3} organisms for reverse transcriptase PCR of 16S rRNA to 10-50 organisms when PCR of the 47-kDa protein gene is used.[36] Some of these assays, such as those targeting the 47-kDa protein gene, may require confirmation using DNA hybridization techniques.[1] In addition, although reverse transcriptase PCR is extremely sensitive, contamination of the specimen by unrelated organisms is a possibility, making a Southern blot step a necessary adjunct for confirmation.[36] Fortunately, newer assays using the *polA* gene have been used on fresh tissue found in genital ulcer specimens from patients with syphilis, with a sensitivity of 95.8% and a specificity of 95.7%, without the need for a confirmatory test.[1,36] However, when PCR targeting of this gene was performed on FFPE cutaneous biopsies, *T pallidum* DNA was detected in only 7 of 36 (19%) cases of syphilis.[37]

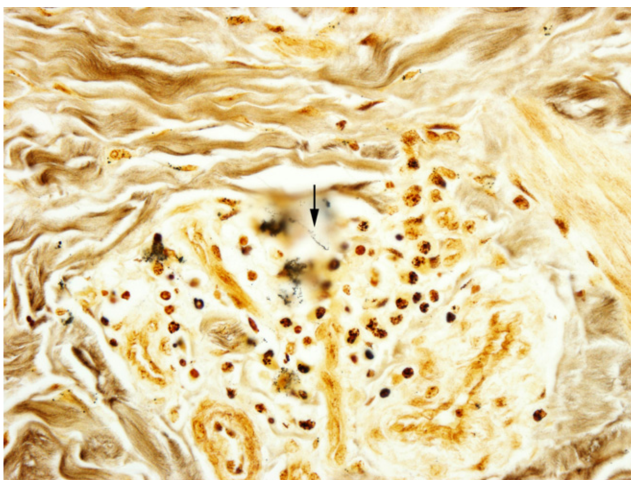

Figure 4 Focal spirochete (under black arrow) at focus of plasma cell–rich dermal infiltrate in skin biopsy specimen from Fig. 3, highlighting the difficulty in identifying organisms using standard silver-stained sections in syphilis (Steiner, ×600).

The authors improved the sensitivity of the assay using a seminested PCR assay targeting smaller fragments of the *polA* gene.[37] In addition to primary syphilis, PCR studies using the 47-kDa protein gene have been successfully used to identify *T pallidum* from FFPE tissue in cutaneous late secondary- and tertiary-stage syphilis.[39]

Rickettsial Disease

Rickettsiae are obligate intracellular bacteria that are divided into the spotted fever group, the typhus group, and the scrub typhus group.[42] Diagnosis is usually clinical, with serologic confirmation. However, seroconversion may take 15-26 days, making serologic diagnosis retrospective in nature and of little use in the acute clinical phase.[43] Isolation in cell culture is difficult and may take up to 60 days for growth, thus limiting its clinical usefulness.[44] In rickettsial disease, early diagnosis is important, as delays in starting antimicrobial therapy is associated with a poor clinical outcome.[45] Skin biopsy with either IHC or direct fluorescent antibody staining is probably the most useful clinical tool in the dermatopathologist's diagnostic arsenal for the confirmation of rickettsial infection. However, these diagnostic tools are not commonly available in most clinical laboratories.

Molecular methods, using PCR, allows for earlier diagnosis and species identification in rickettsial disease. Targeted genes have included the outer-membrane protein genes *ompA* and *ompB*, the citrate synthase gene *gltA*, the 17-kDa protein gene, and 16S rRNA.[46,47] PCR diagnosis of rickettsial disease can be performed on several different tissue sources, including serum, blood clots, eschars, and skin biopsies.[47] In 1 study, 34 of 58 (58.6%) cases of suspected spotted fever rickettsioses were confirmed by nested PCR to detect the *gltA*, 17-kDa protein, *ompA*, and *ompB* genes in skin biopsy specimens.[47] In an attempt to improve the specificity of the PCR technique in the diagnosis of rickettsioses, a method of nested PCR called suicide PCR, which uses single-use primers targeting single-use DNA fragments, has been developed.[43] This particular assay has demonstrated a specificity of 100% and a sensitivity of 68% in the diagnosis of the spotted fever group of rickettsial infections.[43] Although not perfect, molecular techniques at least approach the sensitivity of standard diagnostic adjuncts, such as direct fluorescent-antibody testing (70%), in the diagnosis of rickettsial disease.[48]

Cutaneous Leishmaniasis

Leishmaniasis is a protozoan infection that causes a spectrum of disease, including cutaneous, mucocutaneous, and visceral leishmaniasis, all depending on the geographic location, species, stage of infection, and host response to infection. Identification of *Leishmania* amastigotes, either by direct visualization on tissue smears or FFPE sections or via organism cultivation by culture, remains the gold standard of diagnosis.[49] However, given the variable clinical and histopathologic features of infection that often mimic other infectious or even neoplastic processes, the absence of microscopically identifiable organisms in up to 47% of cases, and the time-consuming and insensitive nature of culture recovery of the organism, molecular testing for leishmaniasis

represents a useful modality to confirm the diagnosis.[49-51] Additionally, unlike direct microscopic identification of the organism, PCR-based assays allow for species identification, which is important for prognosis and determination of the need for therapy.

Identification and speciation of leishmaniasis specimens is carried out by PCR-based testing followed by restriction fragment identification. The PCR assays available include those targeting *Leishmania* ribosomal repeats such as the ribosomal internal transcribed spacer 1 (ITS1) and small subunit ribosomal RNA.[52,53] Other assays include kinetoplast DNA (kDNA), the 7-spliced leader RNA gene, coding and intergenic noncoding regions of the gp63 gene, and the splice leader mini-exon.[53] Of the aforementioned assays, the ITS1 and kDNA assay have been successfully used on FFPE specimens.[49,53,54] Among the ITS1, kDNA, and splice leader mini-exon PCR assays, the kDNA assay showed the highest sensitivity of 98.7%, followed by the ITS1 assay at 91%.[53,55,56] All the PCR assays described earlier in the text have shown nearly 100% specificity for the detection of leishmaniasis.[53]

Fungal Infections

Most of the work performed on the use of molecular methods in the diagnosis of fungal infection has focused on PCR techniques to discriminate between fungi in culture; although it has not focused on the performance of molecular confirmation of fungal infection from FFPE cutaneous specimens.[57] The conventional diagnosis of fungal infection relies on time-consuming fungal cultures, which can take anywhere between 2 and 4 weeks; in some cases, these cultures are of low sensitivity. However, molecular techniques have also been used in the diagnosis of several systemic fungal infections with cutaneous manifestations such as those due to *Aspergillus* spp., *Blastomycosis dermatitidis*, *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Paracoccidiomycosis brasiliensis*.[1] In addition, certain fungal infections that are difficult to diagnose by conventional culture or histopathologic identification of the organism (ie, sporotrichosis) have been confirmed from FFPE tissue specimens using nested PCR assays targeting the 18S rRNA gene.[58] In an atypical cutaneous deep fungal infection in an immunocompromised patient, *Pseudoallescheria boydii* has been successfully identified from FFPE tissue using a seminested PCR assay targeting the genes *Pbo1*, *PboSP1*, and *PboSP2*.[59]

Viral Infections

PCR techniques have been used to detect cutaneous viral pathogens such as herpes simplex virus (HSV), varicella zoster virus, and cytomegalovirus from fresh tissue, blister fluid, and FFPE tissue.[1,60-62] In early or atypical presentations of herpetic viral infection, characteristic viral cytopathic changes may be lacking, making PCR detection of the virus useful.[60,61] Although IHC antigen detection exists for the aforementioned viral pathogens, PCR may in some cases be more reliable, less expensive, and also allow for differentiation between HSV 1, HSV 2, and varicella zoster virus infection.[60-63]

PCR has also been used for the detection and typing of human papillomavirus in nonmelanoma skin cancers, epidermodysplasia verruciformis, and verrucous carcinoma.[1] In addition, although IHC detection of the human herpes virus 8 latent nuclear antigen-1 is the most common method to confirm the diagnosis of Kaposi sarcoma, PCR was historically an important part of differentiating the lesion from other vascular neoplasia.[64] PCR has also been used to identify parapox virus in Milker nodules and ecthyma contagiosum.[65,66]

Conclusions

PCR-based molecular diagnostic assays allow for rapid, sensitive, and specific identification of many pathogens. However, these techniques are associated with high cost, requirements for specialized equipment and specially trained personnel, and, to date, lack of standardization. Therefore, as many of the assays described earlier in the text are not routinely available in the clinical laboratory, conventional methods, including microscopy, culture, and serologic studies, will continue to be of great utility in the diagnosis of many cutaneous pathogens. However, given the limitations of these studies for many fastidious organisms or those infections associated with a small number of organisms, the use of PCR-based molecular diagnostic techniques in the clinical setting will continue to increase in the future.

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