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Detection of bacterial pathogen by Polymerase Chain Reaction (PCR) in case of endophthalmitis

Infectious endophthalmitis despite its being relatively rare, is one of the most devastating complications after ocular surgery or penetrating traumas. It also happened in patients with immunodeficiency or some internal disorders and it is called endogenous endophthalmitis. The most common bacteria which cause acute postoperative endophthalmitis are coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus* spp. In case of delayed endophthalmitis the most common microorganisms are *Propionibacterium acnes*, *Candida albicans,* while in endogenous endophthalmitis there are two main reasons of infection, *Streptococcus pneumoniae* and *Streptococcus viridans*. In the case of penetrating traumas the most common pathogens are *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and fungi (3). It is evident that there are special conditions required for endophthalmitis to occur. It depends on accompanying diseases of the eye, some internal diseases (diabetes, autoimmunological disorders), possibility of occurrence of some complications during and after an ocular surgery. It is obvious that the presence of infectious factor is not sufficient to confirm the diagnosis. Srinivasan and others investigated grade of contamination of the anterior chamber during phacoemulsification and they found the presence of infectious microorganisms in many cases, but none of the patients has developed endophthalmitis (9).

By using conventional microbiological techniques, positive results are obtained, of which merely 0%–76% lead to a clinical dilemma over the cause of the inflammation (4, 7). The difficulties in diagnostic culture appear when it comes to fastidious nature of some of bacterial organisms and small amount of the pathogens in the samples. Other reasons for obtaining negative diagnostic culture are antibiotic preatreatment, inadequate culture methods, or true culture negativity. Many cultures from vitreous or aqueous humor are, in the case of endophthalmitis negative, as well as infections resolved with antimicrobial therapy. A rational approach to the use of antibiotics and steroids necessitates determining whether the inflammation is infectious or sterile. The evolution of new molecular techniques adapted to the field of medical microbiology is a promising tool for the rapid and sensitive identification of bacterial pathogens. These observations suggest that perhaps with more sensitive and specific methods the identification of bacteria in the ocular sample is posssible.

The purpose of this study was to determine the usefulness of PCR method in the diagnosis of endophthalmitis, expecially in the case of culture negative specimens.

MATERIAL AND METHODS

19 specimens (12 aqueous humor and 7 vitreous fluid) were obtained from 13 patients suspected of endophthalmitis, which were treated in 2nd Department of Ophthalmology of Lublin during the period between Dec 2002 and Jan 2005. Those included 7 patients after catarct surgery (4 after extra capsular extraction of cataract – ECCE+ILCP, 3 after phacoemulsiphication Phaco+ILCP), 1 patient after trabeculectomy, 2 patients after penetrating traumas and 3 patients with endogenous endophthtalmitis. 1 patient, which developed endophthtalmitis after cataract surgery, was suffering also from reccurrent uveitis. 5 cases after cataract surgery were classified as acute while 2 cases were classified as delayed. After giving local ansthetic we obtained from 11 patients aqueous humor in quantity 0.05 ml, additionally in 7 patients 0.1–0.2 ml vitreous fluid was obtained during vitrectomy by a syringe connected to the aspiration route. The specimens were delivered immediately to the clinical laboratory.

The samples were analyzed using both conventional microbiological techniques (smear-Gram stain and diagnostic culture) and at the same time PCR directed at 16S rDNA using universal primers was done. Aliquots of clinical specimens were cultured at 37°C for 48 h in glucose broth infusion media, thioglicolat infusion media, Columbia agar (under anaerobic and aerobic conditions), MacConcey agar, Chapman agar and Sabouroud agar at 35°C for 4–5 days.

The process of isolation of DNA was performed according to the protocol commercially available Sherlock kit (DNA-Gdańsk firm). The rule of this method is based on the binding of DNA unusually effectively by using of the ion-exchange membranes. PCR amplification was performed by using universal primers directed on region 16 s rDNA because of the fact that multiple copies of the 16s rDNA genes are present in all bacterial genomes. Details of the primers used in this study appear in Table 1. The size of the final product was 1000 bp (base pair). The total reaction volume was 25 µl containing 5 µl of extracted DNA, 2.5 µl Taq Polymerase 5 ul/ul (DNA-Gdańsk), 5 µl10x PCR buffer, 0.25 µl primers 50 pmol/µl (Invitrogen), 0.25 µl dNTPs 20 mM (Amersham Pharmacia, DNA Polimerization kits) and 17 µl steril distilled water. Amplification was performed in termocycler Cyclone (Biotechnology). The cycling parameters included 5 min at 95°C as an initial step, followed by 35 cycles of denaturizing at 95°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 6 min. For each series of tests a positive control and a negative control was done. Detection of the PCR products was performed on 1% agar's gel containing ethidium bromide by using 10 µl of the amplification mixture and visualized under UV illumination.

Table 1. The primers sequences

Primer 1	5'-ggcggcgcctaaacatgcaagt-3'
Primer 2	5'gacaaccatgcacacctgt-3'

We also investigated the presence of inhibitors in the samples by adding the vitreous or aqueous humor to the positive sample.

RESULTS

Among these 19 specimens 3 (16%) were positive by culture, of which one was *Staphylococcus epidermidis*, the second *Staphylococcus hominis* and the last one *Acinetobacter baumani* compared with 13 positive results obtained by using eubacterial universal primers. Separately in the aqueous

humor the causative pathogen was not identified in 2 cases (17%) by using diagnostic culture compared with 6 cases (50%) by using PCR methods (Fig. 1, Fig. 2). In the vitreous samples the pathogen was identified in 2 cases (22%) by using conventional method compared with 7 cases (100%) by using PCR (Fig. 3, Fig. 4). Microscopic preparation was difficult to evaluate in all samples.

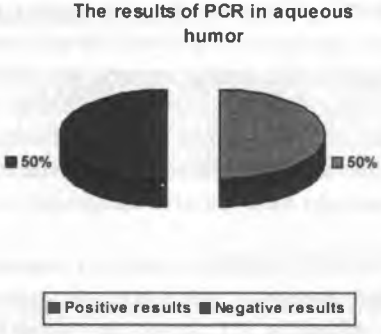

Fig. 1

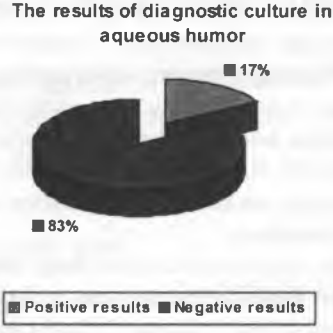

Fig. 2

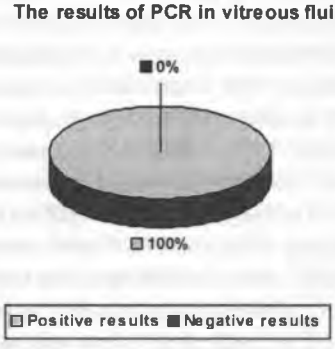

Fig. 3

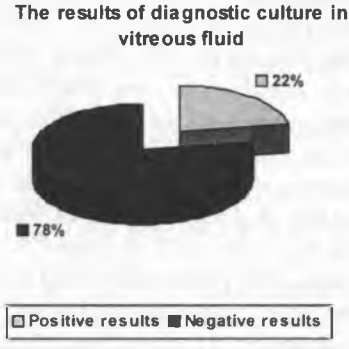

Fig. 4

DISCUSSION

Endophthalmitis is defined as a disease involving at least three eye tissues and post-identification of infectious pathogen (2). Despite this, there are no evidence criteria for classification of frequency of the endophthalmitis. Kresloff and others have estimated endophthalmitis merely after confirming infectious pathogen (3). The other way round, the frequency of endophthalmitis could be minimized, because of the imperfection of diagnostic methods. Identification of infectious factor by using conventional microbiological techniques is very difficult because of a small amount of pathogen in the samples. It is obvious that the most effective treatment is based on rapid diagnosis of the disease and the identification of causative agent, but even though the pathogen is known the treatment of endophthalmitis is very hard because of the difficulty in retaining the high concentration of antibiotics in the vitreous cavity. The next problem accompanying ophthalmic infectious diseases is the growing resistance of the microorganism to antibiotics. There are many papers about resistance of *Staphylococcus* spp to ofloxacin, ciprofloxacyn, cefazolin (6, 10).

Despite the proceedings according to the routine microbiological protocol we obtained 4 (21%), while the other authors 0%–52% in aqueous humor and from 24%–80% in vitreous fluid (1, 4). There are a few reasons which could influence the differences between the results found in the literature and in our paper. The diagnostic culture is maintained up to 48 h for bacteria and 5–10 days for fungi in our laboratory, while in the other one from 5–30 days for all kinds of microorganisms (8). We though that value of the cultures kept for 5–30 days is limited because of finishing the therapy in most of clinical cases. Sandvig and others thought that if the time of diagnostic culture was longer we could obtain the less virulent microorganisms (8). On the other hand, we should remember that the positive diagnostic culture could be a result of contamination. To eliminate false positive results, the same microorganism should be cultured unless on two cultures. It is also possible that aspiration of aqueous humor alone is not sufficient to determine infectious factor. It is known that the vitreous fluid has smaller possibility to clear itself up compared to the aqueous humor.

The usefulness of molecular biological techniques in eye infectious diseases is confirmed by many papers in the world (7). The sensitivity the PCR method in case of endophthalmitis has been estimated for 92% in case of vitreous fluid and 84%–88% in case of aqueous humor. In this paper by using PCR methods we obtain separately100% positive results in vitreous fluid and 50% positive results in aqueous humor. It seemed that aqueous aspirate is insufficient not only for conventional microbiological culture but also for PCR methods.

The process of the extraction of DNA should be carefully chosen to the clinical samples. Sometimes there are situations when the microorganism or DNA may be lost especially when we use some commercial kits (5). Contrary to this, during the method involving simply heating to 95°C as part of PCR- cycling protocol, the DNA could be potentially contaminated by inhibitors of reaction of PCR. We investigated the presence of inhibitors in the samples and we found out one in one sample. In this case it should be better to suspend the specimen in the sterile distilled water or there are possibilities to use anti-inhibitors of PCR reaction.

The staff of the molecular laboratory must be aware that there is a possibility to make some faults during the PCR process. The sensitivity of molecular biological techniques is not available by conventional microbiological methods, but it should be remembered that confirming of the bacterial stain does not equal the confirmation of the etiology of the infection especially where there is no clinical evidence of bacterial process.

CONCLUSIONS

1. PCR performed both on aqueous humor and vitreous fluid is a reliable tool for diagnosis of causative organism particularly in smear and culture negative specimens. The distinction between infectious or noninfectious endophthalmitis plays the main role in successful therapy.

2. Aspiration of aqueous humor is not sufficient in the case of endophthalmitis.

3. The process of extraction of the DNA is the strategic moment in molecular diagnosis.

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SUMMARY

The goal of this study is to determine the usefulness of the PCR method in diagnosing endophthalmitis. 19 clinical specimens 12 AH (aqueous humor) and 7 VF (vitreous fluid) were obtained from 13 eyes with the clinical diagnosis of endophthalmitis. Seven cases after cataract surgery, 1 case of post-trabeculectomy, 2 cases after penetrating traumas, and 3 cases of endogenous endophthalmitis. The same samples were analysed using both conventional microbiological techniques and at the same PCR directed at 16S rDNA using universal primers was done. In the aqueous humor the causative pathogen was identified in one case (5.2%) by using diagnostic culture compared with seven cases (36%) by using PCR methods. In the vitreous samples the pathogen was identified in one case (9%) by using a conventional method compared with five cases (45.4%) by using PCR. Microscopic preparation was difficult to evaluate in all samples. 1. PCR performed on aqueous humor and vitreous fluid is a reliable tool for diagnosis of causative organism, particularly in smear and culture negative specimens. 2. Aspiration of aqueous humor is not sufficcient in the case of endophthalmitis. 3. The proces of extraction of the DNA is the strategic moment in molecular diagnosis.

Diagnostyka zakażeń wnętrza gałki ocznej z użyciem reakcji łańcuchowej polimerazy (PCR)

Celem pracy była ocena przydatności łańcuchowej reakcji polimerazy w diagnostyce zakażeń wnętrza gałki ocznej. Od 13 pacjentów z klinicznymi objawami zakażenia wnętrza gałki ocznej pobrano 19 płynów z komory przedniej i 7 próbek ciała szklistego. Siedem przypadków zapalenia było po operacji zaćmy, jeden po operacji przeciwjaskrowej, dwa przypadki po urazach penetrujących do wnętrza gałki ocznej, trzy przypadki sklasyfikowano jako zapalenia endogenne. Próbki były analizowane jednocześnie z użyciem dwóch metod: konwencjonalnych metod mikrobiologicznych i metodą PCR z użyciem starterów skierowanych na odcinek 16SrDNA. W płynie z komory przedniej przy użyciu konwencjonalnych metod mikrobiologicznych zidentyfikowano bakterię w jednym przypadku (5,2%), podczas gdy przy pomocy PCR stwierdzono obecność patogenów w siedmiu

przypadkach (36%). W ciele szklistym przy użyciu konwencjonalnych metod mikrobiologicznych zidentyfikowano bakterię w jednym przypadku (9%), podczas gdy przy pomocy PCR stwierdzono obecność patogenów w pięciu przypadkach (45,4%). Ocena preparatów bezpośrednich była trudna we wszystkich przypadkach. Skuteczność metody PCR z użyciem uniwersalnego startera bakteryjnego uzasadnia celowość jej zastosowania w diagnostyce zakażeń wnętrza gałki ocznej, zwłaszcza w przypadkach klinicznego podejrzenia zakażenia bakteryjnego, a konwencjonalne metody mikrobiologiczne dają wyniki ujemne. Aspiracja płynu z komory przedniej wydaje się niewystarczająca w przypadku zakażenia wnętrza gałki ocznej. Proces izolacji DNA jest strategicznym momentem w diagnostyce molekularnej.