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Detection of Adenovirus Outbreak at a Municipal Swimming Pool by Nested PCR Amplification

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In July 1995 an outbreak of pharyngoconjuctivitis caused by adenoviruses occurred among athletes participating in a swimming contest in a town in southern Greece (Peloponnese). At least 80 persons displayed symptoms of the illness, with the predominant ones being high fever, sore throat, conjuctivitis, headache, and abdominal pain. Poor chlorination was probably the cause of the outbreak (residual chlorine <0.2 mg/l), as after hyperchlorination the spread of adenoviruses stopped. Rapid detection of adenoviruses in the municipal swimming pool water by nested polymerase chain reaction (PCR) amplification allowed quick control of the outbreak.

Introduction

Adenoviruses are important human pathogens that produce a variety of clinical syndromes in humans. Several studies have reported outbreaks of adenoviral infections in swimming pools.[1–4] The present study confirmed for the first time that the presence of adenoviruses in pool water in Greece was responsible for an outbreak. This is due to the use, for the first time, of a sensitive technique, namely nested polymerase chain reaction (PCR), that has been previously used for the detection of viruses in water.[5–9] Some adenovirus serotypes are difficult to isolate in regular cell lines.[10] As a result of this difficulty certain outbreaks remain unidentified when conventional cell culture techniques are used. These difficulties can be overcome by the use of molecular techniques. PCR is an *in vitro* method based on primer-directed amplification of specific target DNA sequences[11,12] which provide a very rapid, sensitive and specific detection of viruses in different environmental samples.[5,6] Nested PCR amplification is used to ensure the specificity of detection and eliminate false-positive results.[7]

With this method, the detection of adenoviruses in water is obtained in about 36–48 h. In addition, this method enables the detection of ≤ 10 particles of human adenoviruses per sample, a number which is 100 to 1000 times lower than conventional cell culture techniques.[8]

In this study, using molecular techniques we have rapidly detected adenoviruses in swimming pool water. The presence of adenoviruses in these samples confirms that adenoviral infections can be transmitted by swimming pool water.

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Case Report

On 25 and 26 June 1995 a swimming contest with 238 athletes under 18 years old took place in the Matalian swimming pool of Sparti (south Pelloponnese). On 4 and 5 July 1995 symptoms of an infection (high fever 38 °C–39 °C, conjuctivitis, sore throat, weakness and abdominal pains) became evident, and seven of the athletes were hospitalized in the general hospital of Sparti, while another 73 were diagnosed by private pediatricians in Sparti. Both the hospital and private physicians reported that the disease was caused by adenoviruses. However, neither performed virological analyses but based their diagnosis on the clinical symptoms manifested.

Onsets of the illness peaked during 8–15 July, with the outbreak being resolved within 2 weeks. The exact number of the ill athletes is unknown, because those suffering just mild symptoms may not have been reported. It should be noted that although the pool was chlorinated using chlorine gas, the residual chlorine in the water during the outbreak was found to be below 0.2 mg/l; water samples from the municipal pool of Sparti water were examined in order to identify the pathogens responsible for the athletes' illnesses. Moreover, using the same methods we examined the network water to exclude the possibility that contamination of the water from the distribution system (that fills the swimming pool) was the cause of the outbreak.

Laboratory Investigations

An electric 1-horsepower (hp) centrifugal pump was used to collect the sample from the pool. One hundred litres

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of water were collected and filtered through a 1MDS Virosorb filter (AMF CUNO, Meriden, CT, U.S.A.) which eliminates the necessity to adjust the pH of water. The filter was transported to the laboratory and stored at 4 °C. Concurrently 1 litre of the sample was collected for bacteriological analysis.

Before sampling procedure in the laboratory, filter housings were disinfected by soaking for 30 min in buckets filled with water containing more than 5 mg of free chlorine per litre. Hose and pump were disinfected by washing them with water (10 min), bleach (10 min), sodium thiosulfate (10 min) and water (10 min) again.

The viral particles were concentrated by the adsorption-elution method using 1MDS Virosorb filter.[9] The viruses were eluted with 0.25 N glycine buffer, pH 9.5, containing 3% beef extract and the 40 ml volume of viral suspension were recovered and treated as described by Girones *et al.*[7]

In brief, the suspension was centrifuged (48 400 ***g*** for 3 h and 45 min at 4 °C) to form pellets. Viruses retained in the pellet were eluted by the addition of 5 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min and then 25 ml of PBS double concentrated was added to the eluate. The sample was then centrifuged at 12 000 ***g*** for 15 min at 4 °C. The viral suspensions obtained were ultracentrifuged at 171 360 ***g*** for 1 h at 4 °C in order to obtain pellets of the viral particles, which were resuspended in 0.5 ml of PBS and kept at −80 °C until the nucleic acid extraction. A part of this suspension was sent to Dr Annika Allard, Department of Virology, University of Umea, Sweden for the identification of the adenoviruses by culture techniques.

Nucleic acid extraction was processed by Boom's method,[13] with the modifications proposed by Puig,[8] and was applied to the sample. The supernatant with the nucleic acids was kept at −20 °C until the PCR amplification.

The primers used for the detection of adenoviruses were selected from the DNA sequence of the open reading frame of hexon genes of Ad_2, Ad_{40} and Ad_{41} and have been described in previous reports.[7,8,14] The specificity of the general hexon region primers hexAA1885 and hexAA1913 were tested on 18 different adenovirus types representing all six subgenera, and positive results were obtained.[8]

For enzymatic amplification, 10 µl of extracted viral DNA was added to 45 µl of the reaction mix tube containing 20 mM $(NH_4)_2SO_4$, 75 mM Tris HCl pH 9.0, 0.01% (w/v) Tween, 1.5 mM $MgCl_2$, dNTPs (each at 200 µM), each adenovirus primer at 0.08 µM and 2 U of Thermoprime plus DNA polymerase (Advanced Biotechnologies) plus 50 µl of paraffin oil (Sigma) was added. Thermal cycling of the amplification mixture was performed in a programmable heat block (LEP scientific). In all PCR assays the first cycle of denaturation was carried out for 2 min at 94 °C. The conditions for amplification consisted of denaturation at 92 °C for 90 s, annealing at 55 °C for 90 s and extension at 72 °C for 120 s. Thirty thermic cycles were used, followed by a final elongation step at 72 °C for 10 min. External primers were used in the first 30 cycles of amplification and 1 µl (1/50 dilution) of the first PCR reaction was added to a new mix of 49 µl for a PCR containing the nested primer pair nehexAA1893-nehexAA1905 at 0.16 µM each for adenovirus detection. The second PCR reaction (nested PCR) was allowed to proceed for 30 cycles.

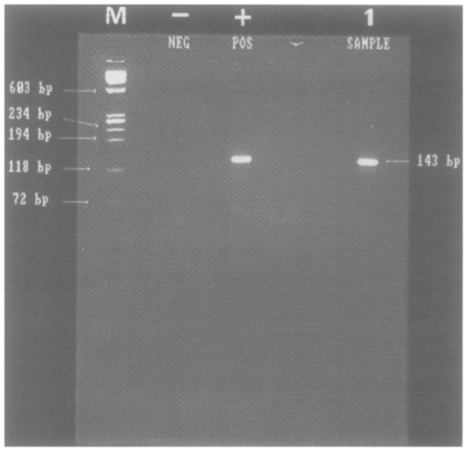

Figure 1. Agarose gel electrophoresis showing amplified product of 143 bases of nested PCR by using adenoviruses primers. Lane M, molecular weight standard marker φX174 HaeIII digest; lane 1, sample from the pool water; lanes − and +, negative and positive controls (adenovirus type 7p). The samples were located in alternate wells to avoid mixing.

A PCR mixture without DNA was used as negative control in each reaction. DNA from adenovirus 7_P was used as a positive control template. Twelve microlitres of the amplification mixture of the first PCR and nested PCR were electrophoresed on a 1.5% Seakem Agarose (FMC Bioproducts) and 3% Seakem Agarose gel, respectively, and photographed under UV light. The product of the first PCR had a length of 301 bp, whereas that from the second nested was 143 bp long. The PCR of the tested sample was found to be positive for adenoviruses (Fig. 1) and negative for enteroviruses and Hepatitis A viruses (figure not shown).

Furthermore, the network water was tested for the presence of adenoviruses, enteroviruses and Hepatitis A virus and was found to be negative.

For the bacteriological analysis the conventional filtration technique was used for the count of total coliforms, faecal coliforms and faecal streptococci. All bacteriological markers were negative.

Discussion

Spread of infectious diseases in swimming pools is well known. Infections reported as having been transmitted in swimming pools include Pseudomonas folliculitis, amoebic meningo-encephalitis and pharyngoconjuctival illness caused by adenoviruses.[1,4,15–17]

Disinfection of swimming pools by chlorination controls the growth of organisms responsible for these infections. It is known that a strong positive correlation exists between free chlorine level and bacteriological quality.[18] Esterman *et al.*[18] reported that residual chlorine in pools at a concentration of ≥ 1 mg/l ensures acceptable bacteriological quality. In the case of the outbreak in Sparti chlorine levels were found to be 0.2 mg/l, well below the threshold for protection, resulting in adenoviral infections. Malfunctioning of the pool chlorination system was probably the cause of the expanded outbreak, as proper operation of the chlorination system prevented further incidents of adenoviral infections. To eliminate adenoviruses we recommend that water is hyperchlorinated (chlorine residual level maintained at 5 mg/l or higher for at least 72 h). Free chlorine residual levels should be 0.6 mg/l or higher at pH 7.2–7.8. This outbreak underlines the importance of adequate chlorination of pool water to prevent disease.

Although not routinely used to detect adenoviruses in pool water, PCR offers two advantages over the conventional cell culture methods, namely rapid recovery time and high sensitivity. Although the techniques used for detection of adenoviruses by PCR cannot distinguish between infectious and non-infectious virions, they provide a means for detecting adenoviruses which do not replicate in cell culture. PCR also overcomes the difficulty of detecting adenoviruses in environmental samples, as it only detects intact nucleic acid sequences. As for cell culture, environmental viral isolates may require several blind passages to adapt to cell culture and produce an observable cytopathic effect.

Apart from the already mentioned ensurance of the specificity of detection and elimination of false positive results, nested PCR amplification also increases the amplification signal. It provides a method with the highest sensitivity and allows the detection of even a small number of viral contaminants in environmental samples.

Traditionally, methods used to isolate viruses from water have relied on animal cell cultures and are technically difficult, time consuming, inefficient and expensive.[19–22] A PCR approach can be used as an alternative to overcome the drawbacks and limitations of conventional techniques.

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