SCIENTIFIC REPORT

Effects of topical anaesthetics and fluorescein on the real-time PCR used for the diagnosis of *Herpesviruses* and *Acanthamoeba* keratitis

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Background: The early microbiological diagnosis of corneal infections may prevent the condition from worsening.

Aim: To study the potential interferences of oxybuprocain and fluorescein solutions used by ophthalmologists on the performances of the real-time polymerase chain reaction (PCR) carried out as routine test for diagnosis of keratitis.

Methods: Quantified suspensions of *Herpes simplex virus* (HSV1), *Varicella zoster virus* (VZV), *Cytomegalovirus* (CMV) and *Acanthamoeba* with and without oxybuprocain or fluorescein added before DNA extraction were tested by real-time PCR.

Results: The capacities of the real-time PCR to detect HSV, VZV, CMV and *Acanthamoeba* were reduced by oxybuprocain and fluorescein. Both products diluted to 1/16 reduced the PCR detection capacities for more than 2 logs (DNA copies/sample).

Conclusions: The simultaneous introduction of fluorescein or topical anaesthetics into the tubes containing the specimens to be tested by PCR may lead to false negative results. Because corneal specimens for microbiological diagnosis of keratitis are obtained after topical administration of anaesthetics and corneal staining with fluorescein, ophthalmologists should be aware to rinse the eye surface intensively with appropriate eye solutions to minimise the risks of misdiagnosis.

Keratitis is an inflammation of the cornea most commonly caused by an infection with bacteria, viruses, fungi or *Acanthamoeba*. Patients with keratitis present with an infected eye and have moderate to severe pain, which may or may not be associated with oedema, cellular infiltration, vascular invasion and scarring, and the treatments depend on the accurate diagnosis of the aetiological agent. Early diagnosis and treatment are essential for improving the visual outcome of patients.¹

Most of the trials comparing the performances of diagnosis of keratitis were conducted using the qualitative polymerase chain reaction (PCR) technology, where the amplified products had to be electrophoresed through 2% agarose and the results assessed as positive or negative by ultraviolet transillumination. The assessment of the end points (visual exam of bands on a gel or immunoassays) does not permit the accurate determination of the partial inhibition provoked by specimens. The development of the real-time PCR technology made quantification of a targeted DNA possible under routine conditions because it created relationships based on the number of cycles elapsed before achieving detectable fluorescence (Ct), and because the threshold is

dependent on the starting target-DNA copy number, the total or partial inhibition of a reaction can be automatically detected if the Ct is delayed.

Because samplings for microbiological diagnosis are carried out after corneal staining with fluorescein or topical administration of anaesthetics, we studied the potential interference of oxybuprocain and fluorescein on the performances of the PCR carried out routinely to detect three different herpesviruses and *Acanthamoeba* associated with eye diseases.

METHODS

Viral or *acanthamoeba* suspensions serially diluted in transport media with or without oxybuprocain or fluorescein were extracted manually by a column-based extraction kit (QIAamp, Qiagen, Courtaboeuf, France) or by the automatic Magnapure total nucleic acid isolation system (Roche, Meylan-Cedex, France).

The primers and labelled probes—the same as those used in the lab for routine diagnosis—were validated with international standards.² Calibrated samples containing titrated viruses purchased from the European Union Concerted Action on Quality Control of Nucleic Acid Amplification Program (EQCP-Glasgow, Glasgow, UK) were tested pure and diluted in distilled water before DNA extraction to assess the sensitivity and detection limits of each assay. Real-time PCRs for each agent were carried out in a final volume of 25 µl containing 2× TaqMan Universal Mastermix (MNL 430449, Applied Biosystems, Courtaboeuf, France), the respective forward primers (0.5 µM), reverse primers (0.5 µM), FAM-TAMRA probes (0.4 µM) and 12.5 µl of the isolated DNAs eluted in distilled water. After incubation for 2 min at 50°C with *uracil N'-glycosylase* (AmpErase, Applied Biosystems, 1 U/100 µl used to prevent the amplification of carryover PCR products by removing any uracil incorporated into single-stranded or double-stranded DNA of potential PCR contaminants) the microtubes were incubated for 10 min at 95°C to inactivate the *uracil N'-glycosylase* and trigger the activity of the AmpliTaq Gold DNA polymerase, (Applied Biosystems). The PCR cycling programme consisted of 50 two-step cycles of 10 s at 95°C and 65 s at 60°C.

The amplification and detection were carried out with the ABI Prism 7000 sequence detector system (Applied Biosystems). Retest and second derivative analysis were carried out with the SmartCyclerII (IL Laboratory, Cepheid, Sunnyvale, California, USA). The Ct value for each sample

Abbreviations: CMV, *Cytomegalovirus*; HSV, *Herpes simplex virus*; IFA, indirect fluorescent antibody assay; NAAT, nucleic acid amplification technique; PCR, polymerase chain reaction; VZV, *Varicella zoster virus*

was determined according to the fluorescence signal exceeding the background limit of 0.20. Each run contained negative controls with no template. The highest peak of the second derivative curves representing the point of maximum curvature of the signal curves, or the transition from non-specific signals and background to amplified product fluorescence, was used to validate the true relevance of the signals.

RESULTS

Table 1 shows the results.

DISCUSSION

Keratitis is a medical emergency and, if not treated, may lead to further injury of the cornea and blindness; the early microbiological diagnosis of eye infections may prevent the condition from worsening to the point of ulceration.

Several trials were published comparing the performances of PCR nucleic acid amplification technique (NAAT) with other laboratory tools used for the diagnosis of keratitis like immunofluorescence (indirect fluorescent assay (IFA)), viral isolation on cell culture, antigen detection or specific immunoglobulin secretion. A survey published in 2003 showed, during a 9-year period, that IFA detected *Herpes simplex virus* (HSV) in 23.7% of the specimens, viral isolation in 9.7% and PCR in 34.6%.³ In 2004, it was reported that the sensitivities of IFA and PCR were equivalent (78.6% and 81.2%) on corneal scrapings from patients with clinically typical dendritic corneal ulcer suggestive of HSV keratitis.⁴ The limited performances found for PCR on corneal samples were recently confirmed by a trial showing that 20.8% of the corneal scrapings obtained from patients with clinically suspected HSV keratitis were positive by viral isolation, whereas 29.2% were positive by PCR and 33.3% by IFA (PCR was positive in all the specimens from which virus was cultured, but only in 82.1% of the specimens in which the HSV antigens were detected).⁵ The sensitivity obtained for PCR on tear samples from patients with stromal herpetic keratitis was 85.7% (compared with the addition of positive viral isolation, IFA and detection of anti-HSV immunoglobulin (Ig) G and 80% if the secretory anti-herpetic IgA was used as the gold standard.⁶ In patients with herpetic uveitis, viral isolation in the aqueous humours was positive in 1%, herpes antigens in 16% and PCR in 25% (15% HSV-1 and 10% *Varicella zoster virus* (VZV)), and the addition of the antigen detection to the positive PCR results allowed a confirmation of the presence of viruses in only 29% of the cases.⁷

For the diagnosis of *Acanthamoeba* keratitis, positive results for PCR were reported for 84% of the corneal epithelial samples studied, whereas culture was positive in 53%, all of

which were PCR positive. Nevertheless, only 71% of culture-positive tear samples were PCR positive⁸, suggesting that the inhibitors present in the tears have not been eliminated during the DNA extraction or purification process. For conjunctival scrapings, Dean *et al*⁹ reported, in more than 1200 samples obtained from individuals scored for trachoma, that the sensitivity of PCR was 90% when the isolation of the bacteria on tissue culture was the gold standard ($\leq 50\%$ of ocular specimens were culture positive after several passages). These low PCR detection levels were confirmed by Kowalski *et al*¹⁰ and by Haller *et al*,¹¹ showing that the commercial Chlamydia PCR test detection capacities were inferior to those obtained after isolation of *Chlamydia trachomatis* on cell culture or direct antigen detection.

The IFA and antigen detection tests are based on the identification of the signals generated by proteins (antigens) expressed by the infectious agent or by the infected cells, and these signals are limited by the stoichiometry of antigen-antibody binding. Surprisingly, in any of the trials previously discussed the detection levels by PCR were considerably higher than those obtained by other techniques. Considering that NAATs detect the equivalent of ≤ 1 organism per assay whereas the limits of detection for conventional methods (IFA, viral isolation, etc) are much higher, the lack of major improvement using the PCRs may limit the interest of NAATs as useful laboratory tools and aids for clinical diagnosis of infections of the eye surface. Hence, if in the previous reports the yield of DNA extracted from the samples and the quality of the specimens (number of cells present in the scrapings) were monitored properly, the molecular diagnosis did not meet the expected goal of enhancing the diagnostic capacities of previous non-molecular-based techniques,³⁻¹³ and the inhibition of the signal amplification process by products used in ophthalmology for the clinical examination (to reduce pain while sampling) has to be considered.

Recently, it was shown using DNA preparations of HSV and toxoplasma that rose Bengal, Lissamine green and calcium alginate inhibit PCR detection,¹² indicating that in the actual ophthalmic context, the molecular diagnosis of keratitis based on NAATs may produce inaccurate results (false negative) and an underestimation of the bacterial, fungal, viral or parasitic load of the cornea. The present work shows that the solutions of oxybuprocain and fluorescein (even diluted 32 times) used in clinical settings inhibit the capacity of the real-time PCR to detect HSV, VSV, cytomegalovirus and *Acanthamoeba*. To minimise the risks of misdiagnosis, ophthalmologists should be aware before sampling that the eye surface should be rinsed intensively with appropriate eye solutions, to avoid the simultaneous introduction of the residual fluorescein or topical anaesthetics into the tubes containing the specimens to be tested by NAATs, and the corneal scrapings to be sent for PCR should be sampled before instillation of rose Bengal or Lissamine green.^{12 13}

Table 1 Results of dilution with oxybuprocain and fluorescein

Agent	Oxybuprocain (dilution)				Fluorescein (dilution)			
	1/2*	1/8*	1/32*	1/64*	1/2*	1/8*	1/32*	1/64*
Herpes simplex 1	I	I	+/-	+	I	I	I	+/-
Varicella zoster	I	I	+/-	+	I	I	I	+/-
Cytomegalovirus	I	I	+	+	I	I	I	+/-
Acanthamoeba	I	I	+	+	I	I	I	+/-

I, inhibition (difference with positive controls >2 log of viral or acanthamoebal DNA copies/reaction); +, no difference between the sample and the positive control (tested without oxybuprocain or fluorescein); +/-, partial inhibition (difference with positive controls ≥ 0.5 but <1 log DNA copies/reaction).

*Levels of inhibition were equivalent for DNA extracted from samples by the automatic Magnapure or the manual Qiagen method.

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