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Collect, boil and amplify – A simple approach for the detection of three common viruses associated with epidemic keratoconjunctivitis, conjunctivitis and dendritic ulcers

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A B S T R A C T

During 2011' an outbreak of epidemic keratoconjunctivitis led to increased clinical requests for molecular screening of viruses from conjunctival swabs. To maximise throughput with minimal cost, a simple boil extraction on dry swabs followed by amplification and real-time detection using 'in-house' assays for herpes simplex viruses (HSV) and adenoviruses with RNaseP as an internal control was validated and introduced. Data from 541 patients who were tested for one or more viral targets was analysed. Adenovirus was most frequently detected accounting for 30% of all cases including the community outbreak. Genotyping of the hexon gene identified the cause as an adenovirus type 8. HSV was detected in 7% of the samples tested, predominantly HSV-1 with a single case of HSV-2. Invalid results due to poor RNaseP signals were reported in 10.5% of samples but for the HSV-1 assay 23% of the samples were invalid due to interference of the fluorescein dye used by ophthalmologists resulting in repeat sampling to obtain a valid result. Despite this, when compared to conventional techniques such as direct immunofluorescence, collect, boil and amplify increased significantly the detection of DNA viruses in conjunctival samples ensuring improved diagnosis, patient management and infection control measures at a modest cost.

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Prior to 2011 the routine laboratory diagnosis of viral conjunctivitis in Wales was performed using virus specific monoclonal antibody direct immunofluorescence. The routine direct immunofluorescence screen included adenovirus, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), Chlamydia trachomatis and varicella zoster virus (VZV). Whilst offering a rapid result, the sensitivity of direct immunofluorescence depends on sample quality and the expertise of the individual reading the slide (Percivalle et al., 2003). Despite an increased use of molecular techniques, direct immunofluorescence remained the method of choice for ophthalmologists throughout Wales and consequently few ocular samples requesting a molecular test were received.

An outbreak of epidemic keratoconjunctivitis characterised by severe bilateral conjunctivitis started during May 2011 within a nursing home for the elderly in South-East Wales. The outbreak rapidly involved the wider community affecting a large region over a protracted time period. As the outbreak continued it became evident that rapid molecular testing was necessary to facilitate appropriate outbreak control measures.

The routine protocol for the collection of an upper respiratory tract sample for respiratory virus investigation by molecular method was by sampling with cotton or flocked tipped swab and transporting it dry to the laboratory (Moore et al., 2008). This method was first used for the collection and transportation of ocular swabs from the community outbreak and then used for the collection of all ocular swabs from other sources including primary care settings and ophthalmology units.

Samples from the original cluster of cases within the nursing home were transported to the Wales Specialist Virology Centre in Cardiff for processing and formal nucleic acid extraction using the NucliSens easyMag (bioMérieux, Marcy-l'Etoile, France) as previously described (Moore et al., 2008). Adenovirus detection was performed on the ABI 7500 FAST using 5 μl of extract in a modified real-time PCR assay based on that described by Heim et al., 2003 (Forward primer 5′GGCACIGTGGGRTTYCTRACTT Reverse primer 5′GCCCCATGTGTCTTACATGCACATC and a locked nucleic acid probe 5′ 6FAM TGC ACC A(+G)A CCC G(+G)R CTCAG BHQ1). The adenovirus assay was duplexed with a human RNaseP assay based on the CDC influenza panel to act as an internal control (Forward primer 5′AGATTTGGACCTGCGAGCG;

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Reverse primer 5′GAGCGGCTGTCTCCACAAGT; Probe 5′NED-TTCTGACCTGAAGGCTCTGCGCG-MGB). The final concentration of each primer in the adenovirus assay was 0.8 μM per reaction with a final probe concentration of 0.2 μM. The final concentration for each primer in the RNaseP assay was 0.4 μM with a final probe concentration of 0.1 μM. The total reaction volume was 25 μl. The assay was optimised for use with the Taqman® Fast Virus 1-step Mix (Applied Biosystems, CA, USA) using the cycling parameters as described in the manufacturers instructions. Using a common crossing threshold (Ct) value of 0.2, a positive adenovirus result was reported when the Ct value of the adenovirus assay was <40, a valid negative result was reported when the RNaseP Ct value was <37 with an adenovirus Ct value of >40 or not detected. Invalid results were reported when the RNaseP Ct value was >37 or not detected.

To test for HSV, either a repeat extraction was performed from the sample Lysis buffer using the EZ1 DSP Virus Kit (Qiagen, Hilden, Germany) or the original EasyMag extract was spiked with the internal control from the artus® HSV-1/2 RG PCR Kit (Qiagen, Hilden, Germany) amplification was then performed on a Rotorgene 6000 and results interpreted using manufacturer's instructions.

To reduce the sample processing and extraction steps of ocular swabs an alternative method was validated. A dry swab 'collect and boil' protocol was already in place testing of genital and lesion samples for HSV. Amplification was performed on a Rotorgene 3000 using an 'in-house' assay targeting the polymerase gene of HSV with common primers but two probes with distinct reporter dyes to distinguish HSV-1 (6FAM) from HSV-2 (Joe) duplexed with a human RNaseP assay (ROX) (unpublished assay, used with the permission of HPA Cambridge laboratory, UK).

The collect and boil extraction protocol was applied to ocular swabs initially from the outbreak. Briefly, swabs were collected and transported dry to the laboratory. On receipt, the swab was immersed into 0.75 ml of 0.01% TE buffer, left to stand for 10 min and vortexed to elute sample material. Two hundred microlitres of TE was transferred and heat treated at 100 °C for 10 min to extract viral DNA. Once pulse centrifuged, 5 μl of extract was used in the adenovirus/RNaseP duplex on the ABI 7500 FAST and 20 μl used in the 'in-house' HSV/RNaseP duplex performed on the Rotorgene 3000.

A prior validation of boiling simulated genital swabs using a method previously described for respiratory syncytial virus (RSV) (Moore et al., 2008) had shown comparable results to formal extraction when tested through the HSV 'in-house' assay (unpublished data). To validate the adenovirus assay, duplicate swabs from 30 outbreak cases were extracted using EasyMag and the boil extraction protocol. There was no significant difference in Ct value between the extraction methods when amplified using the adenovirus/RNaseP assay. Three samples had consistently poor RNaseP signals through both extraction protocols suggesting poor sample quality rather than true inhibition. The validation did not produce any discordant results requiring further confirmation.

Following the validation, all subsequent ocular swabs received in the laboratory from the ongoing outbreak and from other sources querying a viral infection of the eye were tested using the collect, boil and amplify protocol. Using the boil extraction rather than formal extraction procedure there was a significant cost saving of £7.60 per sample based on a local costing model and the time saved to result was approximately 1 h.

From May 2011 until the end of July 2012, a total of 994 ocular swabs from 541 patients were collected and tested for one or

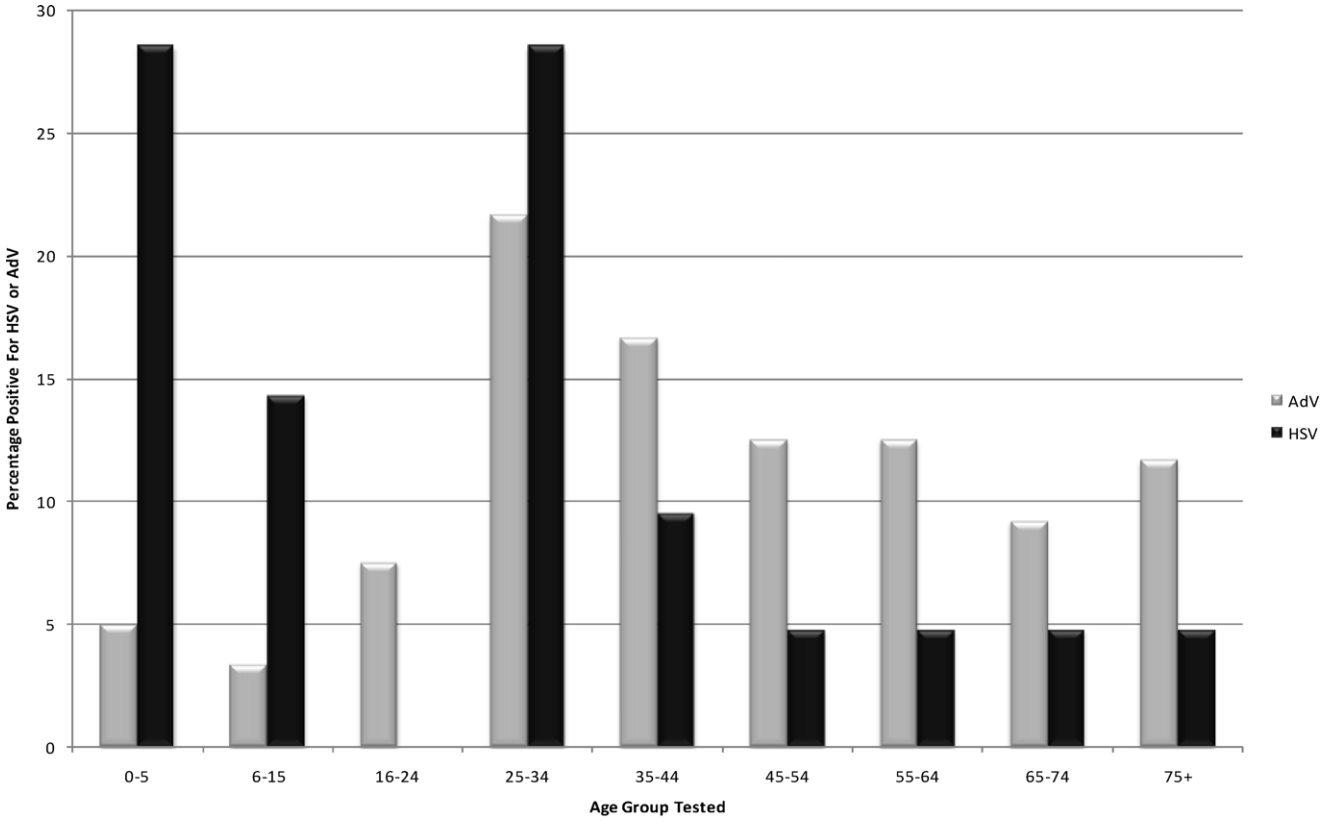

Fig. 1. Positivity Rate by Age Group and Virus. The positivity rate was calculated using the total number of samples tested in each age group for the specific target as a denominator. Adenovirus detections occurred in all age groups peaking in the 25–34 ages. The outbreak of epidemic keratoconjunctivitis occurred in a nursing home for the elderly and exclusively accounted for the >10% of adenovirus cases occurring in the over 75 year old age group. HSV positivity rates peaked in the 0–5 year age group due to the targeted testing of neonates with HSV positive mothers.

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