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The development of a multiplex real-time PCR for the detection of herpes simplex virus 1 and 2, varicella zoster virus, adenovirus and Chlamydia trachomatis from eye swabs

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ABSTRACT

Infectious conjunctivitis can be difficult to distinguish clinically due to the considerable overlap in clinical presentation so clinical diagnosis of conjunctivitis is often insufficient. It is therefore necessary to have a rapid diagnostic test that differentiates between the different causes of infectious conjunctivitis. Screening clinical samples by sample type/syndrome based multiplex real time PCR would allow for rapid detection of a variety of pathogens simultaneously, which will in turn aid in the treatment and clinical management of the patient.

A multiplex real-time PCR assay for rapid and simultaneous detection of HSV 1 and 2, VZV, adenovirus and Chlamydia trachomatis (C. trachomatis) from eye swabs was developed and evaluated. The multiplex assay was shown to be sensitive, specific and robust. Reductions in sample turn around times have been achieved by reducing the amount of separate tests needed to be carried out.

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1. Introduction

Infectious conjunctivitis is common and can be caused by viruses, bacteria, and parasites. Infections are usually mild; however complications can arise and can be sight-threatening.

Infectious conjunctivitis can be difficult to distinguish clinically due to the considerable overlap in clinical presentation so clinical diagnosis of conjunctivitis is often insufficient (O’Brien et al., 2009). This can lead to misdiagnosis resulting in misuse of antibiotics, antibiotic resistance, and subsequently unnecessary costs (Thanathanee and O’Brien, 2011).

It is therefore necessary to have a rapid diagnostic test that differentiates between the different causes of infective conjunctivitis. Traditional detection methods for virus detection rely on often slow, laborious and insensitive cell culturing, which has now been largely replaced by nucleic acid amplification tests. PCR is usually carried out as a single test for each pathogen and tests for all pathogens are not always available at the same site. Screening clinical samples by sample type/syndrome based multiplex real time PCR would allow for rapid detection of a variety of pathogens simultaneously, which will in turn aid in the treatment and clinical management of the patient.

2. Materials and methods

2.1. Assays used in the multiplex

The HSV1/2, VZV and adenovirus assays used in the multiplex were published previously (Ryncarz et al., 1999; van Doornum et al., 2003; Hawrami and Breuer, 1999; Heim et al., 2003). These assays have been shown to be sensitive and specific, and to detect all strains. The C. trachomatis assay was developed in-house (see below).

2.2. Design and evaluation of the C. trachomatis assay

A C. trachomatis assay was designed in-house using primer express™ (Applied Biosystems). Primers and probes were developed to target the cryptic plasmid (sequences given in Table 1). Primers and probes were then compared to all sequences available in BLAST (www.ncbi.nlm.nih.gov/blast) and shown to only detect all serovars of C. trachomatis including the Swedish variant (sw C. trachomatis) discovered in 2006. No interfering
 secondary structures were observed using the mfold algorithm (www.bioinfo.rpi.edu).

2.3. Assessment of the C. trachomatis real time assay

2.3.1. Endpoint sensitivity

The C. trachomatis assay was compared to a fully automated commercial extraction and real-time PCR system, the Abbott RealTime Chlamydia trachomatis/Neisseria gonorrhoea (CT/NG) PCR assay (Abbott, Maidenhead, UK), by comparing endpoint sensitivity using a dilution series of a positive clinical sample.

2.3.2. QC panels

The C. trachomatis assay was further evaluated using the Quality Control for Molecular Diagnostics (QCMD, Glasgow, UK) C. trachomatis proficiency programmes from 2004, 2005 and 2006 and the National External Quality Assessment Service (NEQAS, Sheffield, UK) C. trachomatis distributions 1862 and 1927.

2.3.3. SDA positive clinical samples

182 strand displacement assay (SDA) positive samples consisting of 107 urine samples (66 male and 41 female) and 75 endocervical swabs were tested individually by the real-time PCR assay. Samples had been frozen at -70°C before testing by real-time PCR.

2.4. Assessment of the multiplex assay

2.4.1. Multiplex optimisation

Each probe was labelled with a different fluorescent dye (Table 1); in the case of HSV the HSV 1 and 2 probes were labelled with the same fluorescent dye, atto647. This enabled the detection of all 5 pathogens in one multiplex screen and also reduced the risk of cross-talk between dyes. The inability to differentiate between HSV 1 and 2 was discussed with our users prior to development. Our users agreed it was sufficient to detect both HSV 1 and 2 on the same sample. Optimisation of the HSV probes had to ensure that both probes produced similar levels of fluorescence.

2.4.2. Primer probe optimisation

The primer probe concentrations for all assays, singleton and multiplex, were individually optimised using in-house protocols (Gunson et al., 2003), all primers and probes are shown in Table 1. The VZV probe and all primers where purchased from Applied Biosystems (Cheshire, UK). The remaining probes were purchased from Eurogentec (Seraing, Belgium). The optimised concentration for each probe was 25 μM and for each primer 100 μM.

2.4.3. Endpoint sensitivity

The endpoint detection limits of each component in the multiplex were directly compared to the current routine single assays using a dilution series of positive controls for each target. These were carried out to ensure that multiplexing the assays did not result in a loss of sensitivity at the endpoint of detection.

2.4.4. Specificity

The specificity of the multiplex was further assessed by testing commonly encountered pathogens: human herpes virus types 6 and 7; erythrovirus (parvovirus) B19; norovirus types GI and GII; enterovirus; parechovirus; cytomegalovirus, Epstein barr virus; measles virus; mumps virus; rubella virus; astroivirus; sapovirus; influenza A, B; influenza A H1N1 (2009); influenza A H1N1 (2009) H275Y; coronavirus; 229E, OC43, NL63, HKU1; parainfluenza 1–4; rhinovirus; respiratory syncytial virus types A
Table 2
Endpoint sensitivity of the *C. trachomatis* assay in comparison to the Abbott system.

| Dilution | Abbott CT/NG assay | *C. trachomatis* in-house assay |
|----------|-------------------|-------------------------------|
| Neat     | 27.77             | 26.39                         |
| −1       | 32.02             | 29.90                         |
| −2       | 35.08             | 34.21                         |
| −3       | 37.80*            | 36.30                         |
| −4       | P                 | N                             |
| −5       | N                 | N                             |

* Positive on repeat testing, initial result beyond cut-off.
* Beyond cut-off × 2 = P.

and B; human metapneumovirus types A and B; *Treponema pallidum* (syphilis); *Pneumocystis jirovecii; Mycoplasma pneumoniae*.

2.4.5. Inter- and intra-assay variability

The inter-assay and intra-assay variability of the multiplex was also assessed. The inter-assay variability (reproducibility or long-term precision) was assessed by monitoring positive run controls over 20 PCR runs. This assesses the whole testing system by including extraction and PCR runs with different users. The intra-assay (repeatability or short-term precision) variability was assessed by testing a positive control in 20 wells on one PCR run.

2.5. Laboratory methods

All samples were extracted either on the Qiagen MDx using the QIAamp viral RNA kit (Qiagen, Crawley, UK) or the NucliSens EasyMAG (bioMérieux, Hampshire, UK) according to manufacturer instructions. Both extraction platforms have comparable sensitivity and are used interchangeably. The Abbott commercial CT/NG assay uses the Abbott M2000 automated extraction platform for sample extraction.

PCR was performed on 6 μl of DNA extract with Platinum QPCR PCR mastermix PCR kit (Invitrogen) on an ABI Prism 7500 SDS real-time platform (Applied Biosystems) in a 15 μl reaction volume. The following thermal profile was used: 2 min at 50 °C, 2 min at 95 °C for DNA polymerase activation followed by 40 amplification cycles of 8 sec at 95 °C and 34 sec at 60 °C each (annealing-extension step).

3. Results

3.1. Evaluation of the in-house *C. trachomatis* assay

3.1.1. Endpoint sensitivity

A 10-fold dilution series was assessed through the *C. trachomatis* assay and the Abbott commercial system (Table 2). The results suggest that sensitivity of the *C. trachomatis* assay is similar to the Abbott system, the *C. trachomatis* assay detected down to the −3 dilution, whereas the Abbott system detected the −4 dilution, however this was detected as “beyond the cut-off” without a Ct value. When a sample is detected as “beyond the cut-off” the sample is repeated through the system (extraction and PCR). If the sample is determined “beyond cut-off” on repeat, the sample is reported as positive. The Abbott system detected the −4 dilution as “beyond cut-off” on repeat, therefore these results suggest that the Abbott system may be slightly more sensitive at the endpoint of detection.

3.1.2. QC panels

The *C. trachomatis* assay was assessed using the QCMD *C. trachomatis* proficiency programmes from 2004, 2005 and 2006 and the NEQAS *C. trachomatis* distributions 1862 and 1927. The assay failed to detect one low positive sample (15 copies/ml) in the 2004 QCMD panel. However the assay correctly detected the positive samples in the remaining panels.

3.1.3. SDA positive clinical samples

The assay was also evaluated using a panel of SDA positive clinical samples; the assay detected all 182 SDA positive samples as positive.
3.2. Multiplex evaluation

3.2.1. Endpoint sensitivity

Comparing the endpoint detection limit of each component of the multiplex assay to the single assays showed that multiplexing had no detrimental effect on the endpoint detection limit of each component (Table 3). The adenovirus single assay detected the $10^{-6}$ dilution in 1 out of 2 occasions, the multiplex detected down to the $10^{-5.5}$ dilution. When testing the dilution series of HSV-1, the HSV-1 single assay detected the $10^{-5}$ dilution; the multiplex assay detected the $10^{-5.5}$ dilution in 1 out of 2 occasions. The HSV-2 single assay and multiplex detected the $10^{-6}$ dilution in 1 out of 2 occasions. When testing the dilution series of VZV, the single VZV assay detected the $10^{-6}$ dilution in 1 out of 2 occasions; the multiplex detected the $10^{-5.5}$ dilution. The C. trachomatis assay detected $10^{-5}$; the multiplex assay detected $10^{-5.5}$ in 1 out of 2 occasions.

3.2.2. Specificity

The specificity of the assay was confirmed by testing a panel of other commonly encountered pathogens and no false positive results were encountered.

3.2.3. Inter- and intra-assay variability

The inter-assay variability was assessed by monitoring positive run controls over 20 PCR runs (Table 4a), and intra-assay variability by testing a positive control in 20 wells on one PCR run (Table 4b). The results suggest that there is little inter-assay variability as the CV of each component was similar over 20 different runs with low standard deviation values and low co-efficient of variation (CV) values. The results also suggest that the intra-assay variability of the assay is also good for each component as little variation was observed when the positive control was repeatedly tested and low (CV) values. Overall these results suggest that the duplex assay is precise and robust.

4. Discussion

This paper describes the development and validation of a multiplex real-time PCR assay, which will allow rapid and simultaneous detection of HSV1/2, VZV, adenovirus and C. trachomatis on eye swabs. The validation showed that the newly designed C. trachomatis assay was sensitive and specific. When compared to the Abbott system the in house may be slightly less sensitive at the end-point of detection. The in-house assay therefore may occasionally miss very weak positive C. trachomatis samples. Multiplexing the five assays had no effect on the performance of any of the individual components by assessment of dilution panels. The multiplex assay was shown to be specific by assessment of quality control panels and a panel of commonly encountered pathogens. The multiplex assay was also shown to be precise and robust by assessment of the inter-assay and intra-assay variability.

Multiplexing by sample type/disease syndrome simplifies the routine service; overall costs are reduced when compared to single testing as rapid diagnosis can reduce unnecessary antibiotic usage and investigations. Reductions in sample turn around times have been achieved by reducing the amount of separate tests needed to be carried out. This ensures more samples can be tested within the working day (Gunson et al., 2008). Since the introduction of the real-time PCR multiplex in 2007 the turn around time (per sample) has reduced from an average of 4.5−2 working days (Table 5). In addition the percentage of positive samples also increased since this time. The reason for this increase in detection rate is unclear. It may indicate better sampling by clinicians or could be due to the implementation of the new C. trachomatis real time PCR method.

Multiplex PCR allows for the rapid differentiation between mild and potentially serious causes of conjunctivitis that may be difficult to distinguish clinically. For example, HSV may result in keratoconjunctivitis that is indistinguishable from adenovirus and is the leading cause of viral-induced blindness in the western world. In addition multiplex PCR allows the screening of both viral and bacterial causes of conjunctivitis which can be important as an individual may have to stay off work/school for up to 2 weeks if the infection is viral, whereas in the case of a bacterial infection, such as C. trachomatis, they could return to work/school 24−48 hours after initiating antibiotic treatment.

Although C. trachomatis may not be a serious cause of conjunctivitis, diagnosis is important in aiding management of the patient as it indicates a possible genital infection. The patient will be treated and referred to a genitourinary medicine (GUM) clinic where further testing can be offered. In the case of a baby being infected the mother would also be referred for testing and treatment.

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