Direct in situ reverse transcriptase polymerase chain reaction for detection of measles virus

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Abstract

New methods are described for combined intracellular reverse transcription (RT) and polymerase chain reaction (PCR) using single primer pairs, with direct incorporation of digoxigenin-11-dUTP into amplicons (direct in situ RT/PCR). Routinely used fixatives and minimal pre-treatments were employed. Target sequences of measles virus nucleocapsid (N) and phosphoprotein genes were detected within measles virus infected Vero cells, both in suspension and in formalin-fixed sections, that had been treated by in situ reverse transcription and 30 cycles of direct in situ PCR. Uninfected cells, omission of Taq polymerase, and irrelevant primers were used as controls. Distribution of measles virus within infected cells was determined by in situ hybridisation and immunocytochemistry for measles virus N gene and protein, respectively. Confirmation of amplification within sections was by gel electrophoresis, Southern blotting and sequencing of extracted amplicons. In the majority of cases, measles-infected cells exhibited intense cytoplasmic signal after direct in situ PCR; this was not seen in uninfected cells or infected cells reacted either with irrelevant primers or without Taq polymerase. Unfixed cells in suspension required nested reaction. Measles-specific in situ hybridisation and immunocytochemistry gave an identical signal distribution in sections. Nuclear artifact occurred in some sections and was unpredictable, although it was greatest either in areas of cellular damage, following DNase predigestion, or with vigorous protease pre-treatment. In situ RT-PCR is feasible for measles virus in acutely infected cells both in sections and in suspension. Further work is required to improve the procedure and to eliminate artefactual nuclear signal.

Keywords: Reverse transcription; In situ reverse transcription; Polymerase chain reaction (PCR); In situ PCR; In situ nested PCR; Measles

1. Introduction

Extraction of template nucleic acids for solution phase polymerase chain reaction (PCR), with consequent destruction of morphology, has lim-
ited the use of nucleic acid amplification technology in routine pathology. Ray et al. (1991) and others, Komminoth et al. (1992) and Nuovo (1992) have demonstrated the feasibility of in situ PCR using single primer pairs, and for amplification of short sequences (Ray et al., 1995). Direct in situ PCR, that is, detection of labelled bases incorporated into amplicons, has advantages over indirect detection with in situ hybridisation, eliminating the requirement for specific probes and an additional, complex detection step. However, its specificity has been questioned (Long et al., 1993).

More recently, interest has focused upon in situ PCR combined with prior in situ reverse transcription (in situ RT) for detection of viral RNA in cells (Walboomers et al., 1988; Haase et al., 1990; Krone et al., 1990; Spann et al., 1991; Yap and McGee, 1991; Nuovo et al., 1991; Nuovo, 1992; Bagasra et al., 1992; Embretson et al., 1993), single copy genes (Ray et al., 1991; Komminoth et al., 1992; Nuovo, 1992) and the localisation of mRNA in cells and tissue sections (Chen and Fuggle, 1993; Heniford et al., 1994; Patel et al., 1995).

Major problems remain despite different experimental approaches. A systematic approach embracing pre-treatments, reaction conditions and potential artifact is necessary to provide either improvements or alternatives to existing methodologies.

At the pre-treatment (digestion) step for sections on slides, extensive digestion may compromise morphology, increase target leakage and usually requires a post-fixation step to retain products, thereby precluding a nested (second round) reaction. In contrast, no pre-treatment step appears to be required for alcohol-fixed cell suspensions (Ray et al., 1991).

At the reaction step for slides, the use of concentrated, low volume reaction solutions only allows small samples to be evaluated microscopically (Nuovo et al., 1991). In addition, concentrated solutions may increase the risk of non-specific amplification (Innis and Gelfand, 1990). Current methods do not allow independent confirmation of successful, specific amplification.

This study examined the feasibility of a novel technique for direct in situ RT/PCR on routinely processed specimens using acute measles virus infection as the model.

2. Materials and methods

2.1. Cell and tissue preparations

Acute measles virus infection of cultured primate cells was used as a model, since infection is predominantly cytoplasmic and permits evaluation of the factors that might produce nuclear artifact. Vero (green monkey kidney) cells were cultured either alone or in the presence of Edmondson strain measles virus \(10^7\) plaque-forming units/ml. When at least 80% of infected cells showed syncytial cytopathic effect, cells were scraped into suspension and centrifuged in a 1.5 ml Eppendorf tube at 650 rpm for 1–3 min, and the supernatant discarded. Cells were resuspended in 200 µl phosphate buffered saline (PBS) and centrifuged at 1000 rpm for 1–3 min to a pellet: the pellet was transferred to a 0.5 ml Eppendorf tube and gently resuspended in either 95% alcohol, 10% buffered formalin or PBS alone (unfixed), for 1–10 min. After a further wash in PBS, cell samples were resuspended either directly into the intracellular reverse transcription (in situ RT) reaction mixture, or centrifuged (1000 rpm 1–3 min) onto baked APES 3-aminopropyl-triethoxy silane (APES; Sigma, Poole, UK) coated slides. Slides were air-dried prior to storage at \(-20^\circ C\). Formalin-fixed samples were resuspended in PBS, re-centrifuged (1000 rpm 1–3 min) into a cell block and paraffin-embedded. Additionally, fixed measles-infected and uninfected cells were mixed before embedding.

2.2. Pretreatments for formalin-fixed paraffin sections or alcohol-fixed suspensions

Sections were dewaxed in xylene \((2 \times 5')\) and rehydrated through graded alcohol to \(dH_2O\). Sections were rinsed in PBS 1 mM EDTA and digested in 10 µg/ml proteinase K (Sigma) in PBS/EDTA for intervals of 2–35 min at 37°C. Digestion was stopped by rinsing sections with 0.2% glycine/PBS for 5–10 min, followed by a
Table 1
Sequence of primers

<table>
<thead>
<tr>
<th>Type (negative strand)</th>
<th>Size (bases)</th>
<th>Position</th>
<th>Nucleotide sequence</th>
<th>Size of amplificants (bp)</th>
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</thead>
<tbody>
<tr>
<td>MV1 Sense</td>
<td>21</td>
<td>1198–1219</td>
<td>5'-TTAGGGCAAGAGATGGTAAGG-3'</td>
<td>432</td>
</tr>
<tr>
<td>MV2 Antisense</td>
<td>21</td>
<td>1609–1630</td>
<td>5'-GTTCCTCCCGAGATTCTGC-3'</td>
<td></td>
</tr>
<tr>
<td>MV3 Sense</td>
<td>21</td>
<td>1248–1269</td>
<td>5'-AGCATCTGAACTCGGTATCAC-3'</td>
<td></td>
</tr>
<tr>
<td>MV4 Antisense</td>
<td>21</td>
<td>1479–1500</td>
<td>5'-AGCTCTGCAATCCTTTGCTC-3'</td>
<td></td>
</tr>
<tr>
<td>MRB1 Sense</td>
<td>20</td>
<td>404–419</td>
<td>5'-ATGTTTTATGATCACGCCTGGT-3'</td>
<td>429</td>
</tr>
<tr>
<td>MRB2 Antisense</td>
<td>20</td>
<td>808–828</td>
<td>5'-GGGAAATGATCTTGGATCTTC-3'</td>
<td></td>
</tr>
<tr>
<td>AC1 Sense</td>
<td></td>
<td></td>
<td>5'-CTTTTTCCTGGGCTTTGGATCTTC-3'</td>
<td>202</td>
</tr>
<tr>
<td>AC2 Antisense</td>
<td></td>
<td></td>
<td>5'-CTTTTTCCTGGGCTTTGGATCTTC-3'</td>
<td></td>
</tr>
</tbody>
</table>

MV1–MV4, Measles specific primers, do not detect other morbillivirus sequences.
MRB1, MRB2, Morbillivirus phosphoprotein (P) gene primers, conserved region detects measles and other similar viruses (from Barrett et al., 1993).

PBS wash and air drying. Optimal proteinase K pretreatment conditions were considered to be those which gave most intense specific signal after reaction, whilst preserving morphological detail: 10 min at 37°C was used for subsequent experiments. No digestion was used for cells cytospun onto slides directly from suspension.

2.3. Oligonucleotide primers and probe

Oligonucleotides described previously were used as primers for PCR reactions (Table 1; Applied Biosystems/Oswel, Edinburgh, UK). The riboprobe for measles virus nucleocapsid (N)-gene was described by Cosby et al. (1989). Actin primers AC1 and AC2 (ILS Ltd., Gateshead, UK) were used to amplify a 202 bp fragment from the region 835–1036 of the human β-actin gene (Table 1).

2.4. Solution phase PCR

Total RNA was extracted from infected and uninfected Vero cell cultures by the guanidinium isothiocyanate method (Chirgwin et al., 1979) and recovered by ethanol precipitation. Total RNA was reverse transcribed by methods adapted from Sambrook et al. (1989) and the cDNA amplified by PCR for 30 cycles, with inclusion of digoxigenin-11-dUTP (Emanuel, 1991). Cycling conditions were identical to those used for cells: denature 5 min at 94°C, then 30 cycles of 94°C 1 min, 58°C 2 min, 72°C 1.5 min, followed by 10 min extension at 72°C.

2.5. In situ RT for cells in suspension

All solutions were prepared using DEPC treated water. The reaction mixture used for first strand cDNA synthesis was as follows. To each slide or cell pellet 72 μl of the following was added: 10 μl of 25 mM MgCl₂, 5 μl 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 10 μl each 10 mM dNTP (Pharmacia, St. Albans, UK; or Boehringer Mannheim, Lewes, UK), 2 μl RNase Inhibitor (20 U/μl Boehringer Mannheim), 5 μl 50 μM Random Hexamers (Applied Biosystems, Warrington, UK), 5 μl DEPC-
treated water and 5 µl reverse transcriptase (Moloney murine leukaemia virus 50 U/µl (Applied Biosystems, Warrington, UK) or avian myeloblastosis virus reverse transcriptase (Bioquote, Yorkshire, UK). In some experiments, random hexamers were replaced by either downstream (antisense), control upstream (sense) measles virus primers (4 µl of 15 µM stocks), or oligo dT (5 µl of 50 µM) for the detection of polyadenylated mRNA (β-actin). In all experiments reverse transcription was performed for 2 h at 42°C, and then 5 min at 95°C to destroy the enzyme. For direct incorporation of label into cDNA, 10 µl 10 mM dTTP was replaced by 5 µl 10 mM dTTP and 2 µl of 1 mM digoxigenin-11-dUTP (Boehringer Mannheim).

Following fixation, cell pellets were resuspended in RT mix and two drops of mineral oil were added to each tube before placing on a thermal cycler (Techne PHC-2, Techne Ltd., Cambridge, UK) for the RT step. After RT the mineral oil was removed and an aliquot of cells was taken for gel electrophoresis before the remaining cells were repelleted, and washed in PBS before in situ PCR.

2.6. In situ RT on slides

Sections or cytospun cells were surrounded by a layer of gum (Cowgum, Cow Proofings, Slough, UK) creating a well. The gum was then allowed to set for 5–15 min when a thinner second layer was applied, before adding the RT mixture and sealing the well with a heat-resistant, heat-conducting plastic coverslip cut from the Techne Hi-temp Microplate (Techne Ltd.). Slides were then placed onto the flat cycling block of a thermal cycling machine (Techne Gene E). The temperature parameters and in situ RT reaction components were identical to those used for cell suspensions described below. Following in situ RT, the coverslip and gum were removed, the slides washed in PBS, and air dried prior to PCR, or stored at −20°C.

2.7. In situ PCR for cells in suspension

After in situ RT and PBS rinsing, the cell pellet was resuspended in an isotonic PCR mixture as follows: MgCl₂ 10 µl 25 mM, 10 × PCR buffer 20 µl, each dNTPs 5 µl 10 mM, 1 µl 5 U/µl Taq polymerase (Amplitaq, Perkin Elmer Cetus, CA, USA), 164 µl distilled water, and primers. For single round in situ PCR with in situ hybridisation (ISH), 1–2 µl of each inner flanking primer pair MV3 and MV4 (15 µM) stock, was added (Table 1). For nested PCR reactions, 1–2 µl each outer flanking primer pair MV1 and MV2 (15 µM) stock) was used for the first round PCR, and after centrifugation and resuspension of the cell pellet in PBS, centrifugation was repeated (1000 rpm 1–3 min). The cell pellet was resuspended in an identical reaction mix with inner flanking primers (MV3, MV4) replacing outer flanking primers at identical concentrations. For incorporation of digoxigenin-11-dUTP (Boehringer Mannheim), the 5 µl 10 m dTTP was replaced by 2.5 µl (10 mM) dTTP and 2 µl 1 mM DIG-11-dUTP. After this, two drops of mineral oil were placed onto the surface of the reaction mixture and thermal cycling was performed using the following specifications: initial denaturing 94°C for 10 min; denaturing 94°C 1 min, annealing 58°C 2 min, extension 72°C 1.5 min, 30 cycles; 10 min extension at 72°C. Following thermal cycling, mineral oil was removed and after centrifugation of the cell suspension at 1000 rpm for 1–3 min, the cell pellet was resuspended in PBS after a 20 µl aliquot was taken for either gel electrophoresis and Southern blotting, or sequencing studies. The remainder was either submitted for a second round of in situ nested PCR (where DIG-11-dUTP was incorporated during the second round) or cytospun at 1000–3000 rpm for 1–3 min onto APES-coated slides, and allowed to air-dry before storage at −20°C. Subsequently, either visualisation of incorporated digoxigenin or ISH detection were performed on stored slides.

An osmometer (Camlab, Cambridge, UK) was used to ensure the isotonicity of the initial reaction mixture. Aliquots of cellular suspensions contained 100 000–250 000 cells as measured by a haemocytometer (Marathon Labs, London, UK).
2.8. In situ PCR on slides

A gum-well was prepared as described above, large enough to surround the section. PCR reaction mix contained the following isotonic solution: 20 μl MgCl₂ (25 mM), 40 μl 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3) 10 μl each of (10 mM) dCTP, dGTP, dATP and dTTP (for direct incorporation of label, 5.0 μl dTTP, 2.0 μl of 1 mM DIG-11-dUTP), 2.2 μl of BSA (20 mg/ml molecular biology grade, Boehringer Mannheim), 2–4 μl of each upstream and downstream primer (15 μM) 1.5 μl DNA polymerase and 328 μl of sterile dH₂O. For in situ nested PCR reactions, the DIG-11-dUTP was added in the second round of PCR; the first round reaction mixture contained 10 μl of all dNTPs. Reaction was performed on the flat block of a thermal cycling machine (Techne Gene E) using parameters used for in situ PCR for suspensions. In some experiments, the reaction solution and slides were heated separately; the reaction solution was only added to the slide when the annealing temperature was exceeded ('hot start'). In several experiments, mild physical agitation (every 5 cycles) was performed during reaction.

2.9. Immunodetection of digoxigenin labelled amplificants and hybrids

Sections were quenched in Tris-buffered saline (TBS) containing 10% normal rabbit serum (Dakopatts, High Wycombe, UK) and 3% BSA. Monoclonal anti-Digoxin antibody (Sigma Code D-S 156, Poole, UK) diluted 1:10 000 in TBS was applied for 1 h at room temperature. Following 3 TBS rinses, rabbit anti-mouse IgG (Dakopatts) diluted in 1:25 normal human serum in TBS was applied for 20 min. The slides were then rinsed again in TBS and alkaline phosphatase – anti-alkaline phosphatase (APAAP) (Dakopatts) diluted in 1:25 normal human serum in TBS was applied for 20 min. The slides were then rinsed again in TBS and alkaline phosphatase – anti-alkaline phosphatase (APAAP) (Dakopatts) diluted 1:50 in TBS applied for 30 min. The detection sensitivity was enhanced further by repeating the rabbit anti-mouse and APAAP layers before rinsing in TBS. Alkaline phosphatase was developed using Vector Red alkaline phosphatase substrate Kit 1 (SK-5100, Vector Laboratories, Peterborough, UK). The colour reaction was monitored microscopically before rinsing in TBS, tap water and counterstaining in Carazzi's haematoxylin, dehydrating through alcohol and xylene, and mounting in DPX (BDH, Lutterworth, UK).

2.10. Confirmation of amplificant specificity for measles infected cells in suspension

2.10.1. Gel electrophoresis

After in situ RT-PCR, 20 μl of cell suspension was freeze/thawed 3 times to lyse the cells, and centrifuged at 1500 rpm for 3 min. Twelve microlitres of the supernatant was mixed with loading dye and run on a 2% agarose/TAE gel containing 100 μg/ml ethidium bromide. DNA extracted from paraflin sections following in situ RT-PCR was also analysed by gel electrophoresis for presence of an amplificant band of expected size.

2.10.2. Southern blotting

Following gel electrophoresis, gels were denatured, neutralized and blotted onto nylon membranes (Hybond-N, Amersham, UK) in a 20 × SSC buffer. The filter was probed with a 32P-labelled measles riboprobe, as described above, hybridized overnight at 42°C, then washed in decreasing concentrations of 2 × SSC/sodium dodecylsulphate, air dried and autoradiographed.

2.10.3. Cloning and sequencing

Unlabelled PCR products were precipitated with ethanol and pelleted. For ligation, the DNA pellet was resuspended with 1 μl of the vector pGEM-T (Promega, Poole, UK), 7 μl H₂O, 1 μl × 10 ligase (Promega) and 1 μl T4 ligase (Promega). DH5 α F1 Escherichia coli cells, prepared according to the method of Dagert and Ehrlich (1979), were transformed with the ligation mixture. Transformed cells were plated onto L-agar containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X gal, Sigma) and isopropylthio-β-D-galactoside (IPTG, Sigma) and ampicillin resistant, colonies selected. Colony hybridization was carried out according to the method of Grunstein and Hogness (1975), using a measles virus N-gene probe (pMV-151). Nucleotide sequencing was carried out using dideoxynu-
cleotide chain termination technique with M13 universal primer.

2.10.4. Extraction of DNA from paraffin sections following in situ RT-PCR

Following nested in situ RT-PCR, DNA was extracted from 35 identically treated serial tissue sections using the method of An and Fleming (1991). Parallel experiments were carried out in which one section was examined by ISH using the digoxigenin-labelled riboprobe and a second was removed from the slide and digested in 500 μl of proteinase K solution (5 mg/ml in 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.5% SDS) 24–48 h at 55°C. Total DNA, including cDNA, was extracted by addition of an equal volume of phenol, vortexing and centrifugation (10 000 rpm for 8 min). The aqueous phase was collected and mixed with an equal volume of 1 part chloroform isoamyl alcohol 24:1, 1 part phenol, and centrifuged again. The supernatant was recovered and mixed with an equal volume of chloroform isoamyl alcohol 24:1, re-centrifuged and the upper aqueous phase placed in a sample concentrator (Microcon 30, Amicon, Gloucester, UK) and centrifuged as before. The filter was then rinsed with 300 μl dH₂O and re-centrifuged. The DNA solution was recovered by inverting the microcon filter and centrifuging at 3000 rpm for 4 min. The final volume was usually between 1–5 μl; this was diluted to 10 μl and run on a gel. Even from small blocks of dispersed cells, we were able to recover 10 ng of DNA from one 5 μm section prior to RT-PCR.

2.11. Confirmation of amplificant specificity in formalin-fixed paraffin sections of measles infected Vero cells

2.11.1. Gel electrophoresis and Southern blotting

The extracted DNA and loading dye (5 μl), were resolved on a 1% agarose TAE gel containing 100 μg/ml ethidium bromide at 100 volts. The gel was then photographed, denatured, neutralized and Southern blotted as described above. The filter was probed with a 32P dCTP labelled measles specific PCR product synthesized as follows: 32P dCTP 3.33 μM, dCTP 5 μM; dGTP, dATP, 50 μM; 2.5 units Taq polymerase, 2.5 μM MV₁ and MV₂ primers, 1.5 mM MgCl₂, 50 mM KCl, 10 mM TRIS-HCl pH 8.3 and 100 ng of cDNA prepared from measles infected Vero cell RNA. Thermal cycling was performed using the same conditions as described for in situ PCR. The PCR product was column purified (Clontech Spin 100, Cambridge Bioscience Ltd., Cambridge, UK) and mixed with pre-hybridisation buffer before applying the sample to the filter and undertaking hybridisation at 65°C overnight. The filter was washed twice in 2 × SSC, 1% SDS, for 30 min each at 65°C. The auto radiograph was exposed for 4 days at −70°C.

2.11.2. In situ hybridisation (ISH)

To enable comparison of standard ISH with in situ RT/PCR, ISH was carried out on infected and uninfected Vero cells. A digoxigenin-labelled 186 bp antisense riboprobe was synthesized by standard in vitro transcription methods (Boehringer Mannheim) from a cDNA fragment inserted in transcription vector pGem1 containing the measles nucleoprotein sequences 13755 1561 (Cosby et al., 1989). Cytospins required no pretreatment. Deparaffinized sections of uninfected and infected Vero cells were pre-digested in proteinase K-10 μg/ml for 15–25 min at 37°C, quenched in 0.2% glycine/PBS for 5 min, refixed in 4% paraformaldehyde in PBS for 2 min at 4°C (optional), rinsed in PBS and dehydrated prior to hybridization. The hybridization buffer contained 50% deionized formamide, 5 × SSC, 1 × Denhardt’s (Sigma Ltd.), 10% dextran sulphate (Pharmacia) 0.5 mg/ml yeast tRNA (Boehringer Mannheim), 0.25 mg/ml salmon sperm DNA (Sigma Ltd.) and 10 ng/μl of antisense riboprobe for the detection of amplificants. The buffer and probe were denatured on the slides for 7 min at 90°C, sealed by a glass coverslip, before incubating overnight at 42°C under humid conditions. However, for detection of viral RNA denaturation was omitted. The coverslips were removed by washing in 2 × SSC, followed by two further 15 min washes in 2 × SSC, RNase A 100 μg/ml in 2 × SSC 15 min, 2 × SSC 15 min, then 0.1 × SSC 3 × 15 min (all washes at 37°C). The slides were rinsed in TBS and the presence of digoxi-
Fig. 1. Immunocytochemistry for the detection of measles virus N protein on formalin-fixed sections of mixed infected and uninfected cells. Staining is seen in the cytoplasm of syncitia (all original magnifications × 400).

genin-labelled hybrids detected by the same protocol as used for in situ RT-PCR.

2.11.3. Immunocytochemistry

In order to confirm the presence and distribution of measles virus in infected Vero cells, immunocytochemistry was performed using a monoclonal antibody against the measles virus N-protein (Serolab, Crawley, UK), as described previously (Wakefield et al., 1993).

3. Results

3.1. Detection and localisation of measles virus in cells

By immunohistochemistry, measles-infected Vero cells in suspension and on slides showed an almost exclusively cytoplasmic signal, which was not seen in uninfected cells. On sections of mixed uninfected and infected cells, cytoplasmic signal was found exclusively in areas of syncytia formation (Fig. 1). All samples where primary antibody was omitted, were unstained.

3.2. Indirect in situ RT/PCR for measles virus

Following in situ RT-PCR on alcohol-fixed cells in suspension, an increase in signal was found after ISH in amplified (Fig. 2a) compared with unamplified samples (Fig. 2b).

In sections, infected Vero cells showed a weak, predominantly cytoplasmic signal after in situ RT/PCR and ISH, providing an additional protease step was performed after thermal cycling, prior to ISH. Uninfected cells showed no signal (data not shown).
3.3. Direct in situ RT/PCR for measles-infected cells in suspension: morphological preservation and protease digestion for direct in situ PCR

Cell morphology was well preserved both for cells cytospun onto slides and for cellular sections. Protease digestion (10 μg/ml) of cells in suspension or cytospun onto slides not only compromised morphology, but also dramatically
decreased detectable product on remaining cells (results not shown).

In contrast, cellular sections required a protease digestion step – optimally, 10 min (10 µg/ml). Higher protease concentrations (up to 1 mg/ml) produced poor morphology and increased non-specific nuclear signal. This artefact was also seen in uninfected cells exposed to excessive protease treatment.
Direct in situ RT/PCR performed on alcohol-fixed infected cells produced a prominent cytoplasmic and faint nuclear signal (Fig. 3a). An equally impressive signal was found when nested reactions were carried out (data not shown).

In controls, no signal was seen when reaction was performed on uninfected cells. Similarly, no signal was present when infected cells were thermocycled in a reaction mixture, but with either enzyme, DIG-11-dUTP or primers omitted (Fig. 3b).

3.4. Direct in situ RT/PCR on formalin-fixed paraffin processed Vero cells

In the majority of cases, after direct in situ RT/PCR, measles-infected Vero cells exhibited intense cytoplasmic signal with either faint or absent nuclear signal; uninfected cells showed no signal. For mixed cell populations, cytoplasmic signal was intense in areas of syncytia formation, but uninfected cells were negative (Fig. 4a). This was found both with measles specific primers and with primers specific for a conserved region of the morbillivirus phosphoprotein gene (Barrett et al., 1993). No signal was seen without either Taq polymerase, or DIG-11-dUTP (Fig. 4b). The signal distribution was identical to that seen by immunohistochemistry (Fig. 1).

Occasionally, omission of primers, or addition of irrelevant primers, produced artifactual nuclear signal which occurred in an unpredictable manner. When this artifact was present, nuclear signal was found both within infected and uninfected cells (Fig. 5). ‘Hot start’ (Nuovo et al., 1991) did not reduce the nuclear signal (data not shown). Patchiness of signal was found. This was improved by gentle physical agitation during reaction (data not shown).

DNase I digestion prior to in situ PCR (Fig. 6a), exacerbated the nuclear artifact. It also occurred after nested reaction, when a single PCR cycle with a long extension phase (2 h) was performed, and in areas of nuclear damage (data not shown).

RNase pre-digestion reduced, but did not eliminate cytoplasmic signal following amplification in measles virus infected cells (data not shown). With RNase digestion after in situ RT, a strong cytoplasmic signal was seen, following amplification.

In experiments where deliberate contamination was carried out, uninfected Vero cell sections were bathed in a solution of preformed digoxigenin-labelled amplificants. Peripheral signal around cells and debris was noted (Fig. 6b). This was distinguished readily from ‘true’ cytoplasmic signal.

In situ RT alone with DIG-11-dUTP substituted in the in situ RT, without a subsequent in situ PCR step, produced no signal (data not shown).

3.5. Confirmation of intracellular amplification: gel electrophoresis, Southern blotting and sequencing of PCR products

cDNA preparations of RNA extracted from infected Vero cells showed bands of expected size following tube PCR with the relevant primer pairs (morbillivirus, measles, actin). No bands were visible when either primers or Taq polymerase were omitted.

Alcohol-fixed cells that were lysed after single round in situ RT/PCR using measles primers, showed bands of expected size (Fig. 7a). After the second round of in situ nested RT/PCR, the first round product was markedly diminished and an intense second round product was present (Fig. 7a). Lysis of infected cells before amplification produced no bands, in contrast with those lysed after reaction (Fig. 7b) where appropriately-sized bands were observed. Southern blot analysis confirmed the identity of the amplificant bands after in situ RT/PCR (MV3 and MV4) as specific for measles (Fig. 7c). Sequencing studies on these amplificants confirmed that the sequence was specific for Edmonston strain measles.

DNA extracted from samples on slides after in situ nested PCR showed a faint band of expected size (Fig. 8), in addition to a background blush of larger DNA fragments. The specificity of this band was confirmed by Southern blot analysis (Fig. 8).
Fig. 4. Direct in situ RT/PCR in formalin fixed sections of mixed measles infected and uninfected cells. Infected cells are seen as areas of syncytia formation; the uninfected cells are dispersed (all original magnifications × 400). (a) Direct in situ RT/PCR with measles specific primers. Distribution of signal is similar to that seen in Fig. 1, that is a serial section of the same area. (b) In situ RT followed by thermal cycling in reaction mixture without Taq polymerase enzyme. A similar result was obtained with either omission of primers or addition of irrelevant (CMV) primers.
4. Discussion

A reliable technique for in situ nucleic acid analysis would have a major impact in diagnostic histopathology. We demonstrated that in situ RT/PCR is feasible with fixatives and processes used routinely in diagnostic practice: however, reaction conditions and factors that contribute to artefact require further study.

Acute measles virus infection of Vero cells with specific cytoplasmic signal provided an ideal model for assessing causes of nuclear artefact: this was a feature of direct in situ PCR where it occurred unpredictably, although excessive digestion was a predisposing factor. Artifactual nuclear signal may be due to disruption of nuclear histones and DNA fragmentation during thermal cycling, allowing either non-specific priming, or DNA repair. Signal observed following DNase digestion (where DNA may have been only partially digested), may be due to similar mechanisms. The presence of this artifact with one PCR cycle and long extension phase, suggests repair rather than mispriming is involved.

4.1. Pre-treatment

A mild protease digestion step before in situ RT-PCR was necessary for formalin-fixed sections, but was unnecessary, and even detrimental for alcohol-fixed cells. No detergents were required in our protocol, in contrast to pre-treatments advocated by others (Komminoth and Long, 1993), and no post-fixation step was necessary, thereby permitting nested reaction.

4.2. In situ RT

Since RT efficiency is poor, and evaporation was not a concern during this step, the reaction mixture was not diluted. Prior RNase digestion
Fig. 6. Controls for direct in situ RT/PCR on sections (all original magnifications × 400). (a) DNase digestion before the in situ PCR step. Note artifactual nuclear signal within uninfected cells. (b) Deliberate contamination, adding preformed amplificants to uninfected cells on slides during thermal cycling. Note peripheral cellular rim-like effect and background reticulate pattern which is quite distinct from that seen in Fig. 2b.
greatly reduced, but did not abolish signal completely, suggesting either that RNase penetrates membranes with difficulty or that the reaction is less efficient in intact cells. Interestingly, lysis of cells in suspension after reaction, produced an intense product band in contrast with those lysed...
Fig. 8. (A) shows bands of expected size after solution phase PCR was performed on measles cDNA: lanes 1 and 2 with outer flanking primers MV1 and MV2; lanes 3 and 4 with inner flanking primers MV3 and MV4. (B) After DNA was extracted from 35 sections after in situ RT/PCR (MV3, MV4) genomic DNA blush and faint band of expected size on gel electrophoresis (7252 base pairs), which is clearly seen on Southern blot analysis using a radiolabelled measles-specific probe (C).

before in situ RT/PCR, suggesting that lysis may facilitate the access of degrading enzymes to nucleic acids.

4.3. In situ PCR

Here, relatively iso-osmotic reaction mixtures were used, compared with other protocols (e.g. Nuovo, 1992). Contrary to other methods (Nuovo, 1992), no increase in either Mg$^{2+}$ or enzyme was necessary, and identical solutions were used successfully, for samples both in suspension and on slides. In addition, iso-osmotic solutions may have helped preserve morphology and may have enhanced specificity of reaction in the first few PCR cycles (Innis and Gelfand, 1990). Lack of a complete vapour barrier (mineral oil) permitted limited evaporation and progressive concentration of the reaction components during the amplification phase of PCR which, theoretically, may be of value.

The gum used for slides in these experiments, can accommodate large volumes enabling complete histological sections to be examined. The gum solidifies during thermal cycling and is easily removed after reaction. Although improved specificity has been claimed for 'hot start' (Nuovo, 1992), this was not found to be necessary. Post-reaction fixation (Komminoth and Long, 1993) was unnecessary, and permitted nested reaction without a second digestion step before second round reaction. Gentle agitation of the slide plus reac-
tion solution may reduce the patchiness of signal by causing even distribution of reagent during reaction.

After reaction, sufficient amplified product could be extracted from the large sections used in these experiments, for independent extracellular confirmation of amplification. The reason for the DNA 'blush' of higher molecular weight products by Southern blot is obscure but may represent cDNA or amplified repair products with homology to investigated sequences.

When preformed amplificants were added to the in situ PCR system prior to reaction, in order to produce deliberate contamination, a characteristic signal was observed around the periphery of cells. This was readily distinguished from 'true' signal. The main limitation of the technique — unpredictable artefactual nuclear signal — requires further study, but may in the future be reduced by, for example, labelled primers rather than incorporation of labelled dNTPs.

In conclusion, this model for direct in situ RT/PCR, can produce specific reaction with little background signal and is suitable for both routinely processed cells in suspension and samples on slides. However, vigorous controls and strict reaction conditions are mandatory for the correct interpretation of results. One of the merits of this protocol is that it permits confirmation of successful in situ RT/PCR by independent techniques. Further work is in progress to eliminate nuclear artifact. Nevertheless, direct in situ RT/PCR offers considerable potential, both for diagnosis and for research.

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