Development and validation of laboratory procedures for preimplantation diagnosis of Duchenne muscular dystrophy

Cathy Holding, David Bentley, Roland Roberts, Martin Bobrow, Christopher Mathew

Abstract
In order to develop and validate methods for the preimplantation diagnosis of Duchenne muscular dystrophy (DMD), we have established and evaluated PCR assays for the analysis of four loci within the DMD gene and for two Y chromosome sequences in single cells. A model system using buccal cells picked from mouthwash samples has been used for an extensive evaluation of the sensitivity and specificity of the assays, and each assay has been tested in samples containing single cells, two cells, and three cells per tube. The four DMD and two Y assays have been combined in duplex and triplex reactions to enable simultaneous diagnosis of DMD and of fetal sex. One of the DMD markers is a highly polymorphic simple tandem repeat locus which produces a basic DNA profile, and provides a control for contamination by foreign DNA. Amplification of DMD or Y sequences was observed in 78 to 92% of single male cells, rising to 96% and 97% in tubes containing two or three male cells respectively. Coamplification of both a DMD and a Y sequence together occurred with a mean success of 74% in single male cells, increasing to 93% with two, and 95% with three cells per tube. With appropriate precautions, we believe that it is now possible to proceed to clinical application of these procedures.

The sensitivity of the polymerase chain reaction method (PCR) makes it possible to amplify specific DNA molecules from a single cell. This was first shown by Li et al., who analysed genetic markers from single sperm by probing PCR products with radiolabelled allele specific oligonucleotides in order to generate high resolution linkage maps. Another potential application of this technique is for the preimplantation diagnosis (PID) of genetic disease, by genetic analysis of a blastomere biopsy from an in vitro fertilised (IVF) preimplantation embryo. This strategy of embryo selection offers an important reproductive choice to families who find termination of pregnancy unacceptable, or who have already had several terminations of pregnancy after prenatal diagnosis of a genetic disorder. Handyside et al. showed that single blastomeres could be sexed by amplification of a Y specific sequence which is present at 500 to 8000 copies per cell. Nested PCR, a procedure using an outer pair of PCR primers for an initial series of cycles, and an inner pair for subsequent reamplification, provided the increased sensitivity and specificity necessary to detect and analyse single copy sequences in single cells.

Early reports of the clinical application of these techniques have appeared based on removal of a single cell from eight cell stage embryos or on the analysis of polar bodies. However, diagnostic errors have occurred in two of 10 pregnancies established after preimplantation diagnosis. A fetus diagnosed as female was subsequently found to be male by prenatal diagnosis and the pregnancy was terminated, and a fetus diagnosed by blastomere biopsy as unaffected with cystic fibrosis was shown by prenatal diagnosis to be affected.

It is a tribute to the sensitivity of available techniques that single cell genetic diagnosis can even be considered, but if these procedures are to move into regular clinical practice, it is essential to establish the reliability with which single gene defects can be detected, and to design assays with adequate safeguards against technical error in diagnosis. Diagnostic accuracy is improved if: (1) results always depend on the presence rather than the absence of a particular band; (2) more than one locus can be tested to reinforce the results; and (3) simultaneous checks can be run for contamination by foreign DNA.

We have developed such systems for the preimplantation diagnosis of Duchenne muscular dystrophy (DMD). The ethical and practical difficulties of testing large numbers of human blastomeres have led us to develop a model system for validation of PCR assays using single buccal epithelial cells. This has the advantage of relatively easy sample collection from persons of known genotype.

In order to provide secure information on DMD genotype, fetal sex, and DNA contamination, it is necessary to test more than one locus simultaneously. We have evaluated PCR amplification of four different single copy sequences (PERT87-15, PERT87-8, exon 50, STR50) in the X linked DMD gene, which contain polymorphic DNA markers that are commonly used in carrier detection and prenatal diagnosis of DMD, or are markers for intragenic deletions which occur frequently in DMD patients. One or more of these sequences were amplified simultaneously with
either a single copy or a repetitive copy Y chromosome sequence, which could be used to determine the sex of the embryo. We asked whether the Y repeat sequence reduced the efficiency of amplification of the single copy X sequences, and examined the relative efficiencies of the different Y specific reactions in diagnosing sex. We have assessed the use of X markers together with Y specific PCRs, so that female sex is diagnosed by the positive presence of a specific paternally derived X marker, rather than by the absence of a Y marker, which is indistinguishable from amplification failure. We have evaluated coamplification of two single copy X sequences together, and in conjunction with the Y repeat sequence (that is, three simultaneous PCRs), to determine whether the amplification of the X specific sequences could act as controls for each other and for the Y repeat PCR. We have tested a multiallelic CA repeat marker, STR50, which, in addition to being a highly informative diagnostic marker, provides a basic DNA profile and will be of value in detecting contamination.

We have determined the reproducibility of these assays performed on one, two, or three cells per tube, using buccal cells from donors of known genotype. Our results define the limits of reliable detection of single copy sequences for application to the preimplantation diagnosis of DMD and other monogenic defects for the techniques current in this laboratory. We confirm that preimplantation diagnosis can be sufficiently accurate for clinical application, provided that appropriate counselling and back up prenatal diagnosis are available. Whether it will generally be acceptable depends on the safety of embryo biopsy, which has yet to be extensively assessed, and on the acceptability of the IVF procedure to this group of patients.

### Materials and methods

**PREPARATION OF CELLS**

Male buccal cells were used in these evaluations. Amplifications of DMD and Y chromosome sequences are based, therefore, on DNA from a single chromosome. Buccal cells were obtained by gently scraping the inside of the cheek with a clean wooden applicator stick at least 30 minutes after consuming food or drink. The stick was gently twirled in a petri dish containing 5 ml sterile phosphate buffered saline (PBS). Single cells, or groups of two or three cells, were removed from the petri dish using a finely drawn Pasteur pipette, and washed in a second petri dish also containing 5 ml sterile PBS. Cells were removed using a second finely drawn Pasteur pipette into sterile 0.75 ml tubes, containing 1 ml sterile PBS. To each tube was added 1 μl of 17 μmol/l SDS (sodium dodecyl sulphate) and 2 μl of 125 μg/ml proteinase K. The tubes were incubated at 37°C for 60 minutes, then 99°C for 15 minutes, centrifuged for 15 seconds in a microfuge, and then held at room temperature during preparation of reaction mixes for PCR.

**AMPLIFICATION OF DMD AND Y LOCI BY PCR**

The four DMD loci amplified were as follows: pERT87-15B and pERT87-8T contain BamHI (B) or TaqI (T) restriction fragment length polymorphisms (RFLPs), are 47% and 41% heterozygous, respectively, and are retained in DMD patients. STR50 is a CA repeat in intron 50 with a heterozygosity of 71-6%, and is thus of value as a marker for PCR contamination as well as for linkage analysis. STR50 is deleted in 10-6% of all DMD patients. Exon 50, the most frequently deleted exon in the DMD gene, is deleted in 17-5% of all DMD patients.

The YS2 sequence (amplified product 115 bp) is a single copy sequence located on the Y chromosome close to the pseudoautosomal boundary. The PCR assay for repetitive sequences on the Y chromosome (product size 170 bp) amplifies a region close to that described previously.

PCR primers for the primary and nested amplification of these loci were designed from available sequences as follows. pERT87-15B and pERT87-8T: Koenig et al, Roberts et al, Beggs et al, and from sequence data derived by R Roberts (GenBank accession numbers L05931 and L05532). STR50: Clemen et al, and from sequence data derived by R Follen. YS2: Ellis et al and N Ellis (personal communication). Y repeat: Nakahori et al.

The primer sequences for the four loci are listed in table 1. All primer pairs were tested on genomic DNA from persons of known genotype before use in single cell amplifications.

- **PCR** was carried out in 50 μl reaction mixes using Cetus GeneAmp kit components as directed by the manufacturer. Alternatively, reactions were carried out in other commercially available buffers with final concentrations of...
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1-5 mmol/l MgCl₂, 200 μmol/l deoxynucleotide triphosphates (dNTPs), 1 μmol/l of each PCR primer, and 0.05% of Nonidet P-40 (NP40). A master mix of these reagents was prepared sufficient for each experiment, according to the Cetus instructions, except for the addition of 2-5 μl of 1% NP40 and 1-25 units of Taq DNA polymerase for each reaction mix.

A total of 45 μl reaction mix containing a mixture of outer primers was added to each tube containing the lysed cells, by pipetting the liquid down the side of the tube from the top. No other mixing was carried out. One drop of mineral oil (Sigma light white in 6 ml dropper bottles) was overlaid on each reaction mix and the tubes placed in a thermal cycler. Cycle conditions were set at 93°C for one minute, 58°C for one minute, 72°C for three minutes for 30 cycles, followed by extension at 72°C for five minutes. An annealing temperature of 40°C in place of 58°C was used for the multiplex amplification of pERT 87-15B, YS2, and STR50.

Following 30 cycles of amplification, tubes were held at room temperature, usually overnight. Reaction mixes were separated from oil into sterile lidded microtitre dishes. Reamplification reaction mixes were prepared and dispensed on ice, overlaid with mineral oil, and then 1 μl of the first amplification mix was added to separate second reaction mixes containing inner primer pairs for each locus, and mixed by gently flicking the tubes. Tubes were reamplified for 30 cycles exactly as previously described, except for STR50, which was reamplified as follows: 93°C for one minute, 55°C for one minute, 72°C for three minutes for 20 cycles only.

**ANALYSIS OF PCR PRODUCTS pERT87-15B and pERT87-8T**

Second round amplification products (10 μl) were digested with 10 U of BamHI or TaqI respectively in a final volume of 30 μl at 37°C for two to four hours in buffers supplied by the manufacturers. Digestion products were electrophoresed at 60 mA for 20 minutes in 4% NuSieve agarose gels containing ethidium bromide and visualised under ultraviolet light.

**STR50 and exon 50**

PCR product and alleles could be visualised in NuSieve gels containing ethidium bromide. In order to obtain optimal resolution of all of the STR50 alleles, the PCR products were radioactively labelled and electrophoresed in a sequencing gel as follows. During the second round of amplification, one internal primer was end labelled using [γ-³²P] ATP. ϒ²² PCR was carried out using 1 pmol of the labelled primer and 10 pmol of the second, unlabelled internal primer per reaction mix. Products were electrophoresed in 5% denaturing acrylamide gels at 40 W for 1.5 hours, fixed, washed, dried, and autoradiographed. Alleles were scored after one to five hours.

**YS2 and Y-repeat**

Products from the second (YS2) or first (Y repeat) PCR were separated from oil, and 10 to 15 μl electrophoresed in 4% NuSieve gels as for pERT87-15B and pERT87-8T.

**CONTROLS FOR PCR CONTAMINATION**

The degree of contamination in the cell collection and PCR procedures was assessed as follows. (1) The cell collection procedure was mimicked, using only PBS in the absence of cells; and (2) PCR reactions were dispensed and subjected to amplification without any other manipulations. Control tubes which contained only reaction mix were included in each experiment.

**PRECAUTIONS AGAINST CONTAMINATION**

The set up area for preparation of the tubes containing the cells was in a separate laboratory from that used for PCR. Cells were handled and reaction mixes prepared in a class II laminar flow hood. Autoclaved plugged tips were used for aliquotting the 1 μl PBS into sterile tubes, for preparation of master mixes, and for aliquotting the reaction mixes into tubes. Full sterile handling precautions were observed. PCR was performed and PCR products were separated from oil in the main laboratory. Amplified samples were never taken into the set up laboratory. A separate set of automatic micropipettes was used for reamplification, and these were taken apart and UV irradiated for 15 to 20 minutes before use in each experiment. Sterile unplugged tips were used, but these were purchased pre-racked. Tips were used once only and discarded. A wet tip was never placed in the master mix. After reamplification, reaction mixes were separated from oil using a third set of untreated micropipettes and laboratory stock pipette tips.

**Results**

Examples of the electrophoretic analysis of the products of coamplification of DMD and Y sequences are shown in fig 1. Nested amplification of single copy DMD and Y sequences produced a single clear band of the expected size from most tubes which contained one or two male buccal cells. The repetitive Y sequence, which was analysed after only a single round of amplification, showed the expected PCR product and a band resulting from primer–dimer formation (fig 1C and D, and Handyside et al). In addition, the results in fig 1 show that failure of amplification of a single copy sequence can occur independently of amplification of a second sequence in the same cell. For example, in fig 1A (track 6) the pERT87-15B locus amplified successfully, whereas no signal was obtained from the YS2 locus. This cell would therefore be typed incorrectly as female in origin. In fig 1B (track 5) it is the 87-15B reaction that has failed, whereas the YS2 locus has been amplified. The results of coamplification of DMD
RFLPs with Y chromosome sequences in one, two, or three male cells per tube are shown in table 2, based on 686 individual amplification experiments. Successful amplification of both a diagnostic marker for DMD and a Y sequence for fetal sexing occurred at a rate of 74% for single cells, rising to 93% and 95% for two and three cells respectively. The success rates for the two DMD markers were equivalent whether they were coamplified with a single copy Y sequence or with the Y repeat. The individual amplification efficiencies for the three single copy sequences within the duplex reactions (table 2) were very similar, with a range of 78% to 81% in single cells, and approached 100% when two or three cells were used in each reaction. The Y repeat reaction was significantly more robust than the single copy reactions at the single cell level (92% ± 79%), but had a similar efficiency in samples which contained two or three cells. The data from table 2 can be used to estimate the proportion of male single cells in which fetal sex would be incorrectly diagnosed as female. A total of 20/134 male single cells were positive for the X reaction and negative for the single copy Y reaction (YS2), giving an error rate of 14.9%. This fell to 2/160 cells, or 1.3%, with the Y repeat. The rate of misdiagnosis of sex in samples containing two or three cells was less than 1% for the Y repeat, but 2.3% and 4.8% for the single copy Y reaction.

Amplification efficiency was also examined in two different triplex PCR amplifications. In the first triplex, exon 50, which is one of the exons which is most frequently deleted in the dystrophin gene,9 was added to the pERT87-15B and YR reactions. The data in table 3 shows that coamplification of this third locus does not have a significantly adverse effect on the YR reaction, although the efficiency of the pERT87-15B reaction is somewhat lower.

Simultaneous amplification of all three loci occurred at a relatively low rate in single cells, but at high rates with two or three cells. The efficiency of amplification of the highly polymorphic CA repeat marker within intron 50 of the DMD gene (STR5014) was also examined both as a single marker and in a triplex reaction with pERT87-15B and YS2 (table 4). The
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<table>
<thead>
<tr>
<th>Table 3</th>
<th>Results of triplex amplification (exon 50, pERT87-15B, and YR) in male buccal cells.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cell/tube (n = 58)</td>
<td>2 cells/tube (n = 40)</td>
</tr>
<tr>
<td>All 3 alleles amplified</td>
<td>46</td>
</tr>
<tr>
<td>2 out of 3 amplified</td>
<td>23</td>
</tr>
<tr>
<td>1 out of 3 amplified</td>
<td>23</td>
</tr>
<tr>
<td>None amplified</td>
<td>8</td>
</tr>
</tbody>
</table>

Amplification efficiency

<table>
<thead>
<tr>
<th>pERT87-15B</th>
<th>Exon 50</th>
<th>YR</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>50</td>
<td>89</td>
</tr>
<tr>
<td>97</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Results expressed as percentage of tubes giving a reaction. n = number of tubes tested.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Results of amplification of the CA repeat marker (STR50) in male buccal cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A STR50 alone</td>
<td>1 cell/tube (n = 70)</td>
</tr>
<tr>
<td>2 cells/tube (n = 39)</td>
<td>97%</td>
</tr>
<tr>
<td>B Triplex (STR50, pERT87-15B, and YS2) in single male cells (n = 50)</td>
<td>All three alleles amplified</td>
</tr>
<tr>
<td>2 out of 3 amplified</td>
<td>20%</td>
</tr>
<tr>
<td>1 out of 3 amplified</td>
<td>8%</td>
</tr>
<tr>
<td>None amplified</td>
<td>16%</td>
</tr>
</tbody>
</table>

Amplification efficiencies

<table>
<thead>
<tr>
<th>STR50</th>
<th>pERT87-15B</th>
<th>YS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>74%</td>
<td>72%</td>
<td>70%</td>
</tr>
</tbody>
</table>

Results indicate that STR50 is not affected by the presence of the other two loci, and can be amplified with an efficiency similar to conventional single copy loci. The value of this marker in detecting contaminating DNA is shown in fig 2.

The results of experiments to test for contamination of the PCR are shown in table 5. Mimicking of the cell collection procedure with PBS produced one false positive in 188 PCRs (0.5%). No false positives were detected in 214 PCRs from reaction blanks.

Discussion

This study shows that PCR of single copy loci in the DMD gene and from the Y chromosome in a large series of single buccal cells does not consistently produce successful amplification in more than about 70 to 80% of tests. Our results are in agreement with those of Monk et al., who detected both alleles of the β globin gene with an efficiency of 71% in single buccal cells, and Verlinsky et al. who obtained success rates of 78% and 83% in polar bodies and blastomeres respectively. Amplification efficiencies of 95% or greater have been reported for the cystic fibrosis locus in single spermatozoa, and for the GR globin and parathyroid hormone gene loci, also in spermatozoa.25 It is possible that amplification success rates may be tissue or locus specific, perhaps because of differences in chromatin structure, with the limiting factor for PCR being the removal of all chromosomal proteins from the target sequence. Preliminary results from a limited number of single human blastomeres (C Holding and S Pickering, unpublished data) showed that the pERT87-15B locus was amplified in 23 of 26 male cells, but a larger series will be required to establish whether success rates in blastomeres are significantly different from buccal cells. In contrast to the results for single copy sequences, amplification of the Y repeat from single cells produced success rates in excess of 90% after a single round of PCR.

Amplification from two cells increased our success rates to about 93%, and with three cells to about 95%. The use of two cells per assay rather than a single cell would therefore markedly improve the efficiency of the diagnosis. There is evidence that two cell biopsies in mice26 and in humans10 are compatible with continued short term in vitro development of an early embryo. A judgement will have to be made between the greater diagnostic accuracy of using larger biopsies and any increased hazard to the embryo.

Analysis of the failure rates of individual reactions within duplex and triplex assays does not suggest a significant degree of interference of one assay with another (tables 2, 3, and 4). This will be particularly helpful in the context of preimplantation diagnosis for DMD, since it allows for the diagnosis of fetal sex as well as for typing more than one marker in the DMD gene, which has a high frequency of intragenic recombination.27 The fact that coamplification of the Y repeat sequence does not affect amplification of single copy DMD loci is also encouraging, given the greater sensitivity of this assay for the diagnosis of fetal sex. However, the probability of detection of all of the alleles from two or more loci in single cells is relatively low. The coamplification rates are approximately equal to the products of the success rates of individual reactions, reinforcing the conclusion that the different amplification reactions proceed more or less independently.

Product from one locus does not guarantee product from any of the other loci present in the same cell: the Y specific reaction, whether it was from the single or multiple copy sequence, failed on occasion in single cells, for example in fig 2A (lanes 6 and 9), even though the X specific reaction succeeded. However, this also occurred when three sequences were amplified simultaneously (tables 3 and 4). Since each reaction at the single cell level appears to be proceeding independently of any others in the same tube, internal PCR controls are not reliable indicators of amplification of the test sequence at this level of sensitivity.

The data from tables 2, 3, and 4 can be used to assess success rates and error rates for the diagnosis of fetal sex and of DMD. Of particular concern would be the misdiagnosis of a male embryo as female as a result of failure of amplification of the Y sequence. The greater sensitivity of the Y repeat reaction, and the fact that it does not interfere significantly with the X reactions, suggests that it should be used in preference to single copy Y sequences for sexing blastomeres. The error rate for the Y repeat in single cells (that is, male cells with a X−Y− genotype) is 1 to 2%, which falls to less than 1% for two or three cells. The consequences of a false positive result from the Y reaction are less significant and are likely to be
rare provided that adequate safeguards against contamination are applied. In the absence of significant contamination, half of the embryos from an IVF procedure could therefore be diagnosed as female, and hence unaffected, with a high degree of accuracy. In male embryos, both the X and Y loci will be amplified in about 75% of single cells (table 2). Since half of the male embryos, on average, from a DMD carrier will be affected, 37.5% of male embryos could be typed as unaffected if diagnosis was based on biopsy of a single cell. Thus for every 10 embryos, an average of six or seven would be diagnosed as unaffected. This figure rises to an average of seven or eight if two cells are used for the analysis, and the accuracy of the diagnosis of fetal sex would be significantly increased.

This assessment of diagnostic accuracy is based on the assumption that contamination of the blastomere biopsy with spermatozoa, cumulus cells, or cells from the operator can be prevented or detected, and that the PCR reactions do not become contaminated with amplification products from other sources. Provided rigorous protocols, designed to avoid contamination of the reactions with spurious PCR products or operator cells, are followed (see Materials and methods), false positives from these sources occur at a very low frequency (table 5). Contamination of the blastomere biopsy with paternal or maternal cells could be detected by analysis with highly polymorphic microsatellite markers. If both parents, and the laboratory personnel involved, are genotyped beforehand with several such markers from the DMD gene, a marker could generally be found which allowed the two maternal alleles and the paternal allele to be distinguished. We have shown this with a single X chromosome simple tandem repeat locus (fig 2). However, table 4 indicates that the amplification efficiency of STR50 is only 74% when amplified in conjunction with pERT87-15B and YS2, and so, in theory, a single contaminating cell in the embryo biopsy could only be identified three out of four times. In two cells, as with conventional loci, its amplification efficiency is very high.

In conclusion, our results suggest that two blastomeres from a single embryo biopsy would be necessary and sufficient for reliable preimplantation genetic diagnosis. Diagnosis could be achieved by testing a single blastomere, but the rate of technical failures and of misdiagnosis would be significantly higher. In general, these results give an encouraging view of the potential for accurate preimplantation diagnosis of monogenic disorders, but we believe that the following principles should be adhered to. (1) No diagnosis should be based on the absence of a result, but always on the presence of an amplified sequence. (2) Whenever possible, more than one diagnostic test should be applied simultaneously, as a cover for error or failure of one of the reactions. (3) Rigorous tests for DNA contamination should be included with all samples, and wherever possible included in the specific diagnostic assay itself. (4) All patients should be properly counselled beforehand, and confirmatory prenatal diagnosis undertaken later in pregnancy. (5) All continuing pregnancies should be followed up for some years after birth, to confirm the safety of the biopsy procedures.

We thank Rachel Flomen for the provision of STR50 sequence data, and Elizabeth Manners for her assistance in the preparation of the manuscript. This work was supported by The Wellcome Trust, The Generation Trust, The Muscular Dystrophy Group of Great Britain and Northern Ireland, and The Spastics Society.

![Figure 2. Amplification of CA repeats in intron 50 (STR50). Detection of contamination of samples containing two male buccal cells with one female buccal cell, with a different number of CA repeats. Track 3 shows amplification of one allele of contaminating DNA in the absence of amplification of the sample DNA. M = male pattern; F = female pattern; O = operator (CH) pattern.](Image)

**Table 5** 402 contamination controls pooled from all experiments.

<table>
<thead>
<tr>
<th>Type of blank</th>
<th>Total No of blanks assayed</th>
<th>pERT87-15B</th>
<th>YS2</th>
<th>YR</th>
<th>pERT87-8T</th>
<th>No of expts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free PBS</td>
<td>94</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Reaction mix only</td>
<td>77</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

Results expressed as percentage of tubes positive for PCR product.
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