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Why There Is no Link Between Measles Virus and Autism

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1. Introduction

A report published in the Lancet in 1998 described the case histories of 12 previously normal children who developed symptoms of autism or inflammatory bowel disease after having received the measles, mumps, and rubella (MMR) vaccine [1]. This paper formed the basis for Andrew Wakefield's subsequent claim to have identified a new type of gastrointestinal disease, termed autistic enterocolitis. Despite never explicitly asserting a link between the MMR vaccine and this supposedly new, regressive form of autism, the paper sparked a major health scare in the United Kingdom. It is probable that the uncertainty and controversy surrounding the relationship between measles and autism contributed to the fact that in 2004/05, about 1.9 million school children and 300,000 pre-school children were recorded as incompletely vaccinated against measles in England, including more than 800,000 children completely unvaccinated. Based on this, approximately 1.3 million children aged 2-17 years were susceptible to measles [2]. In 2006, a 13-year old boy, who had not received the MMR vaccine, became the first person in the UK for 14 years to die of measles and as a result of almost a decade of low MMR vaccination coverage across the UK, by 2008 the disease had once again become endemic.

In 2010 the Lancet fully retracted the 1998 publication from the public record, stating that it had "become clear that several elements of the 1998 paper by Wakefield *et al* are incorrect, contrary to the findings of an earlier investigation". The circumstances surrounding this publication were subject to an extensive investigation and received a huge amount of publicity. Wakefield was found guilty of serious professional misconduct over the way he carried out his research and was struck off the medical register in 2010. A long statement released on 24 May 2010 includes the following key statements:

- “In all the circumstances and taking into account the standard which might be expected of a doctor practising in the same field of medicine in similar circumstances in or around 1996-1998, the Panel concluded that Dr Wakefield’s misconduct not only collectively amounts to serious professional misconduct, over a timeframe from 1996 to 1999, but also, when considered individually, constitutes multiple separate instances of serious professional misconduct. Accordingly the Panel finds Dr Wakefield guilty of serious professional misconduct” and

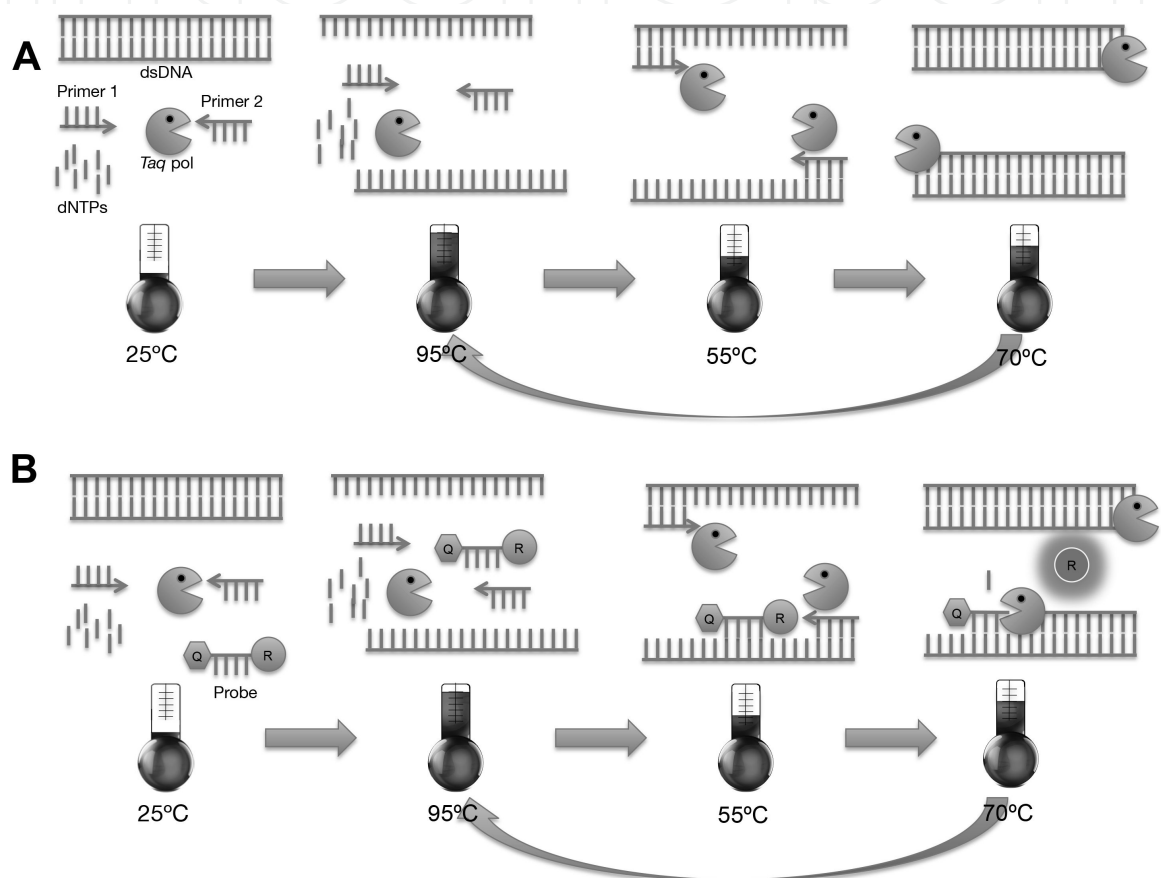


Figure 1. The polymerase chain reaction, a method for copying the same stretch of DNA several million-fold. **A.** A PCR reaction consists of double stranded DNA, two short DNA molecules (“primers”) whose sequence is complementary to opposite strands of the DNA, a DNA synthesis enzyme (“*Taq* polymerase”) and four nucleotide building blocks (“dNTPs”). The reaction mixture is heated to 95°C to dissociate the sample DNA strands, then cooled to around 55°C to allow the two primers to bind to their targets on the individual strands. Next, *Taq* polymerase makes two new strands of DNA at its optimal temperature of around 70°C, using the original strands as templates, hence duplicating the original DNA. This procedure is repeated many times, leading to more than one billion exact copies of the original DNA segment. These can be detected by running the samples on a gel and staining with a DNA-binding dye. **B.** qPCR obviates the need for gel electrophoresis by using fluorescence to detect copied DNA. The qPCR method used for the detection of MeV uses a target-specific DNA molecule (“probe”) that has a fluorescent dye at one end (R) and a quencher (Q) on the other. In the absence of target, the quencher prevents the dye from emitting light. In the presence of target, the probe binds to its target and is degraded by the *Taq* polymerase. This separates the fluorescent label and the quencher and so results in the emission of light. Both the PCR and light detection are automated and detected in a single step by a dedicated instrument.

- “Accordingly the Panel has determined that Dr Wakefield’s name should be erased from the medical register. The Panel concluded that it is the only sanction that is appropriate to protect patients and is in the wider public interest, including the maintenance of public trust and confidence in the profession and is proportionate to the serious and wide-ranging findings made against him” [3].

There was far less publicity about the attempts to use molecular techniques to corroborate a link between measles virus (MeV) and “autistic enterocolitis”. The major technique used was the fluorescence-based real-time polymerase chain reaction (qPCR), a ubiquitous technique used for the sensitive and specific detection of DNA (Figure 1).

2. Technology and target

2.1. qPCR

A key attraction of qPCR technology is its apparent simplicity: an assay consisting of combining oligonucleotides, PCR enzyme and buffer with a nucleic acid template to produce a qPCR reaction is perceived as undemanding. This practical simplicity is complemented by the absence of any requirement for post-assay handling, as well as the development of user-friendly data analysis software that makes data generation and visualisation in the shape of amplification plots remarkably simple. Indeed, qPCR is often described as a mature technology and as the “gold standard” for nucleic acid quantification. Whilst it is true that the technology is capable of exquisite sensitivity and specificity, coupled with high reproducibility and accuracy, it is essential to understand that qPCR assays are made up of numerous, often divergent protocols that use different instruments, enzymes, buffers and non-identical targets.

Whilst qPCR is the method of choice for the detection of DNA, the enzyme (*Taq* polymerase) used to copy the DNA template does not work well with RNA. Hence, if the amplification target is RNA, as it is for MeV, an enzymatic RNA-to-DNA conversion (reverse transcription, RT) step must be carried out before the DNA copying step can specifically amplify the target of interest. This variant of the qPCR is termed reverse-transcription (RT)-qPCR and the principle of this reaction is simple: RNA is reverse transcribed into single stranded DNA, either in a separate reaction or as a “one tube” assay that uses a different enzyme (*Tth* polymerase), as was the case with the experiments discussed below. There are several detection chemistries, but in this case a very specific and widely used probe-based method was used (Figure 1B). Importantly, the use of fluorescent reporter molecules permits concurrent target amplification, detection and quantification as the assay proceeds [4]. Fluorescence is detected using dedicated qPCR instruments, which have (i) a controllable heating block that can hold a variety of temperatures and rapidly change between them, (ii) an excitation light source to excite the fluorochrome, (iii) a detector to register photon emissions and (iv) software that allows analysis of the data. Fluorescence emissions are collected from each sample tube and the levels of background fluorescence detected by the fluorimeter module of the qPCR instrument are established, providing a baseline that defines the sensitivity of the in-

strument. Instrument-specific algorithms are used to define a fluorescence threshold for each sample. Finally, the algorithm searches the data from each sample for a point that exceeds the baseline and plots a characteristic amplification plot.

Increases in fluorescent signal are proportional to the amount of DNA produced during each PCR cycle and produce a characteristic quantification cycle (C_q) for every test. As a result, the more initial target there is, the sooner the instrument can detect the fluorescence and the lower the C_q. Conversely, a higher C_q denotes less initial target. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range in the presence of suitable standards.

The consistency and reliability of RT-qPCR assays depends on the appropriate execution of a number of steps, principally those involving sample selection, template quality, assay design and data analysis.

2.2. Measles virus

MeVs, from the family Paramyxoviridae, genus Morbillivirus, have a single negative-strand RNA genome enclosed in a viral envelope associated with three proteins: the matrix (M) lining the inner surface of the envelope and the fusion (F) and haemagglutinin (H) transmembrane proteins. Negative sense means that the RNA is complementary to mRNA and must be copied into the complementary plus-sense mRNA before proteins can be made. Thus, besides needing to code for an RNA-dependent RNA-polymerase, these viruses also need to package it in the virion so that they can make mRNAs upon infecting the cell. The MeV RNA-dependent RNA polymerase generates a full-length positive copy of their genome, the “replicative intermediate” as well as individual RNA copies that serve as mRNA for individual virus-specific proteins (Figure 2). Importantly, at no stage of its replication cycle is MeV RNA ever reverse transcribed into DNA, *i.e.* MeV does not exist as a DNA molecule, a fact that was established in 1964. This is a critical issue, since if it can be demonstrated that a test is amplifying DNA and not RNA, this provides indisputable proof of contamination.

2.3. What makes a publication credible?

Before describing the experiments in detail, it is worth reiterating what is expected of a plausible scientific publication. Its credibility depends on a number of conditions that include

- Transparency of reporting
- Reliability of protocols and techniques
- Inclusion of appropriate controls
- Reproducibility of the data

This is especially so for publications that utilises qPCR, since its sensitivity makes experiments susceptible to contamination, which leads to the reporting of false positive results.

The purpose of this chapter is to demonstrate that the qPCR data claiming to detect MeV in the intestine of autistic children are unreliable and meaningless because of

- Absence of transparency: the key publication shows no data; hence an expert reader cannot evaluate the reliability of its conclusions
- Unreliable techniques and protocols: analysis of the qPCR data was incorrect
- Disregard for controls: obvious evidence of extensive contamination was disregarded
- Lack of reproducibility: the data could not be duplicated by several independent investigators

The only conclusion possible is that the assays were detecting contaminating DNA. Since MeV is an RNA-only virus and never exists in DNA form, these data must be ignored and it is my opinion that the authors should withdraw this publication from the peer-reviewed literature.

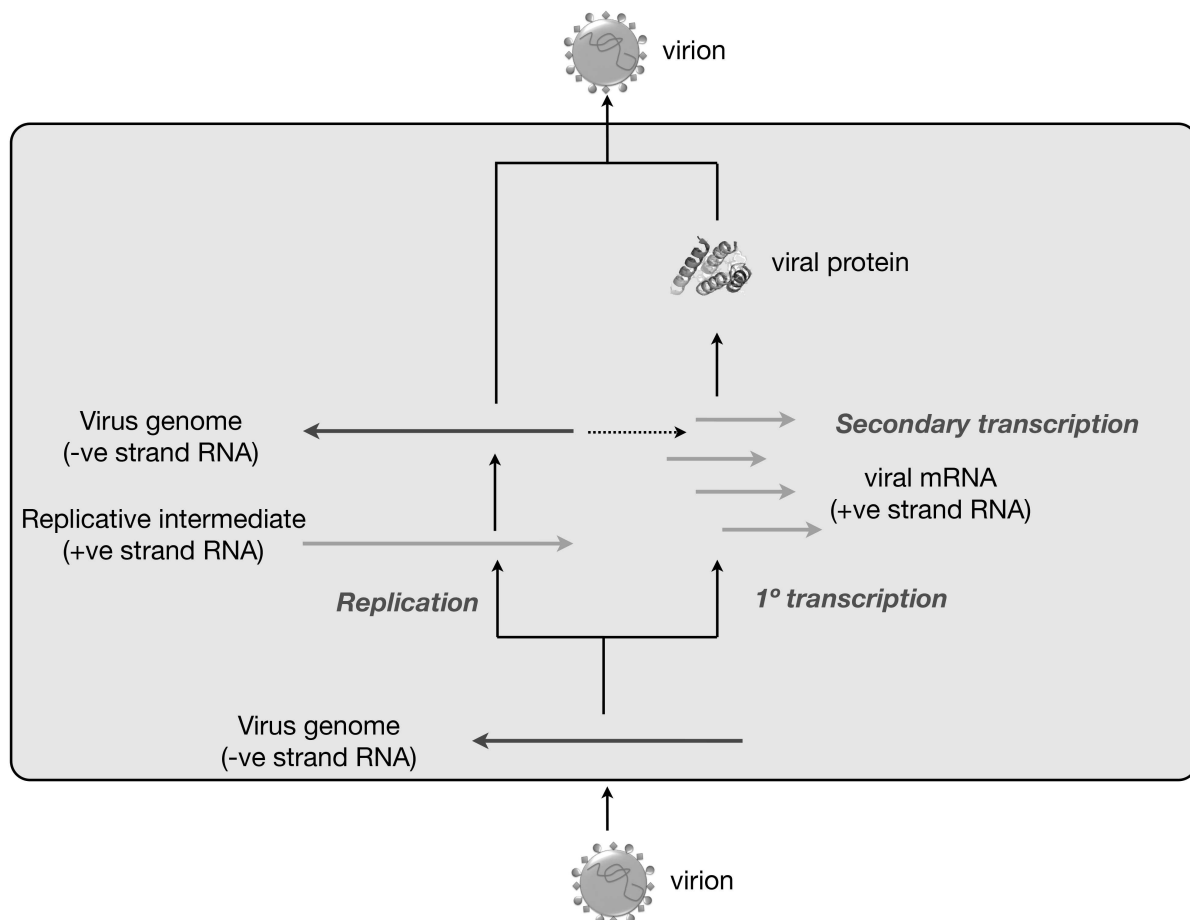


Figure 2. MeV life cycle. The virus attaches to the surface of a host cell, the viral envelope fuses to the plasma membrane and the nucleocapsid is released into the cell. Negative-sense genomic RNA is transcribed into individual messenger RNAs as well as a full-length positive-sense RNA template, which is used to create negative-sense RNA. Viral proteins are translated, assembled around the negative sense RNA and new viruses bud from the cells.

3. Timeline

3.1. April 2000

An Irish pathologist, John O'Leary, reports "scientific results in a series of children with autistic enterocolitis... following an approach made by Andrew Wakefield" before the US Congress Committee on Government Reform [5]. He had "compelling" evidence "in relation to the presence of measles virus in children with autistic enterocolitis" and could "confirm that his [Wakefield's] hypothesis is correct". This statement was based on the detection of MeV using the then rather novel reverse transcription (RT)-qPCR assay as well other methods, in 24/25 "children with autistic enterocolitis", compared to 1/15 control children. O'Leary also emphasised that he went to "desperate lengths" to prove the absence of contamination problems to "outrule the possible generation of false positives". Importantly, he stressed that "nothing in [his] testimony should or must be construed as anti-vaccine; rather it encourages safe vaccine strategies". This final qualification, although very clear, was buried by the headline news of the link between MeV and "autistic enterocolitis".

3.2. August 2000

A brief letter signed by JJ O'Leary, V Uhlmann and AJ Wakefield appeared in the Lancet [6]. It asserted that their "data from molecular virological studies examining the role of measles virus infection in children with autism and enterocolitis have been peer-reviewed, presented, and published at four international scientific meetings". The letter contained references to other publications, with reference 4 listing a publication by Uhlmann *et al.* entitled "Identification of measles virus genomes in ileo-colonic lymphoid hyperplasia in children" as "in press" in the Journal Laboratory Investigations. However, there is no record of such a publication.

3.3. April 2002

Speculation about a possible association between intestinal abnormalities in children with developmental disorders and the MMR vaccine was encouraged by a publication that utilised RT-qPCR assays to screen children's intestinal biopsies for the presence of MeV [7]. A comparison of terminal ileal samples from 70 normal controls and 91 children with a "new form of developmental disorder, ileocolonic lymphonodular hyperplasia", led to the claim that whereas 75/91 of the affected children patients tested positive for MeV, only 5/70 control patients did. The paper does not reveal whether the autistic children had been given the MMR vaccination, but in the context of the source (Royal Free Hospital), authors (including Wakefield), introduction (reference to Wakefield's paper) and the discussion one is left with the impression that they had. The prominently displayed "take home message" concludes "the data confirmed an association between the presence of measles virus and gut pathology in children with developmental disorder". These results, if true, would constitute hard evidence linking MeV, gut pathology and autism and was indeed used to support the vaccination/autism theory, even though the authors themselves never made that specific link.

3.4. May 2004

An abstract entitled “TaqMan RT-PCR Detection of Measles Virus Genomic RNA in Cerebrospinal Fluid in Children with Regressive Autism” and published on the website of the Association of American Physicians and Surgeons lists two of the authors from the 2002 publication as co-authors, with JJ O’Leary occupying the senior author position[8]. It reports the detection of MeV in the cerebrospinal fluid of 19/28 children presenting “as autistic regression closely following MMR vaccination” compared with 1/37 controls. In five cases a haemagglutinin[*sic*] gene allelic discrimination assay was carried out and showed a result consistent with a vaccine strain being detected. The abstract concludes with the sentence: “The findings confirm a highly significant statistical association between the presence of MV RNA in CSF and autistic regression following MMR vaccination”.

3.5. Summer 2004

A paper is published that includes six patients (three patients, three controls) whose data were already published in the May 2004 abstract. It reports the detection of MeV in 3/3 patients but 0/3 controls [9]. Interestingly, O’Leary and Sheils are not listed as authors; instead the publication only thanks them in the acknowledgements.

3.6. June 2007

The first of three omnibus autism proceedings took place at the US Court of Federal Claims, where all vaccine claims are managed and are adjudicated by the Office of Special Masters. The trials were designed to establish whether or not autism could be caused by thimerosal containing vaccines, by MMR vaccine, or a combination of the two. I acted as an expert witness for the US Department of Justice (DoJ), presenting evidence based on earlier reports I had prepared for the UK High Court, who gave permission for the release to the US Secretary of the Department of Health and Human Services of two reports authored by myself. These documents had been filed by the three principal defendants in the UK MMR vaccine litigation and constituted an exhaustive analysis of the raw data underlying the results reported by the 2002 O’Leary publication. The evidence presented to the trial is a matter of public record, with the transcript available from the autism omnibus proceedings[10].

3.7. February 2009-August 2010

The evidence presented at the 2007 trial was used in further trials that concluded in February 2009 that there was no credible link between the MMR vaccine and autism. These decisions were upheld on appeal in July/August 2009 and then again in August 2010 were read [11].

4. Analysis of the 2002 O’Leary paper

The analyses and conclusions shown below were reached after examining all the RT-qPCR data disclosed following a court order. New experimental reports were generated using

identical analysis software, compared with those disclosed by the O'Leary laboratory and any differences were noted. Any ambiguous or discordant results and all results involving negative controls were further investigated by scrutinising the raw data collected by the qPCR instrument. This permitted a definitive resolution of all ambiguities. All disclosed operator sheets and laboratory notebook entries relevant to the RT-qPCR assay were read and compared with the disclosed and the reanalysed data. Standard operating procedures were examined and inconsistencies with actual procedures were noted.

1. Transparency of reporting

The purpose of publishing a paper in the peer-reviewed literature is to provide adequate information that allows any competent scientist to follow the published protocol and reproduce the published data. Hence it is essential that detailed descriptions of the methods used and of the results obtained are included. It is not acceptable to publish summarised results only without any supporting, relevant data.

- a. RNA was extracted from fresh frozen samples as well as formalin fixed, paraffin-embedded tissue (FFPE). However, there is no information on how the fresh samples were frozen, how long they had been stored, what percentage of patient and control samples were fresh frozen or FFPE and whether the same percentage was in each category. This is essential, since it is well established that FFPE treatment modifies and destroys RNA, or in Prof O'Leary's own words "wax and fixation by itself breaks down RNA" [5]. Hence it was well known at that time that RNA-derived data obtained from FFPE samples must be analysed and interpreted with caution [12-14].
- b. No information is provided with respect to quantification or quality assessment of the extracted RNA; indeed there is no mention of RNA quality. This is vital information needed to assess the validity of any quantitative or negative result [15].
- c. The RT-qPCR results are summarised without providing any actual data; a table simply states that 70/91 children with gut pathology were positive for MeV, as against 4/70 in the control group.
- d. MeV copy numbers in the affected children are reported as ranging from 1 to 3×10^5 copies of RNA/ng total RNA; no corresponding figure is provided for the four positive samples from the control samples. There is also no indication of what the potential error in those copy numbers might be.
- e. Despite claims that the authors looked at two viral gene targets, they used only the data from the F-gene, which were discordant with the H-gene results.

2. Reliability of protocols and techniques

- a. RNA was extracted from both fresh frozen and FFPE tissue samples and subjected to RT-qPCR analysis. Two RNAs were targeted; a control mRNA specified by the GAPDH gene and the MeV F-gene. As discussed earlier, since FFPE samples are characterised by RNA degradation, the expectation is that the results obtained from the FFPE samples should be different and, in terms of quantification, there should be less RNA present in

a FFPE sample. This is in fact the results the authors obtain for the control RNA, where there was an approximately 4,000-fold reduction in RNA levels (Figure 3A). In contrast, the results recorded for MeV RNA were the same regardless of its source (Figure 3B).

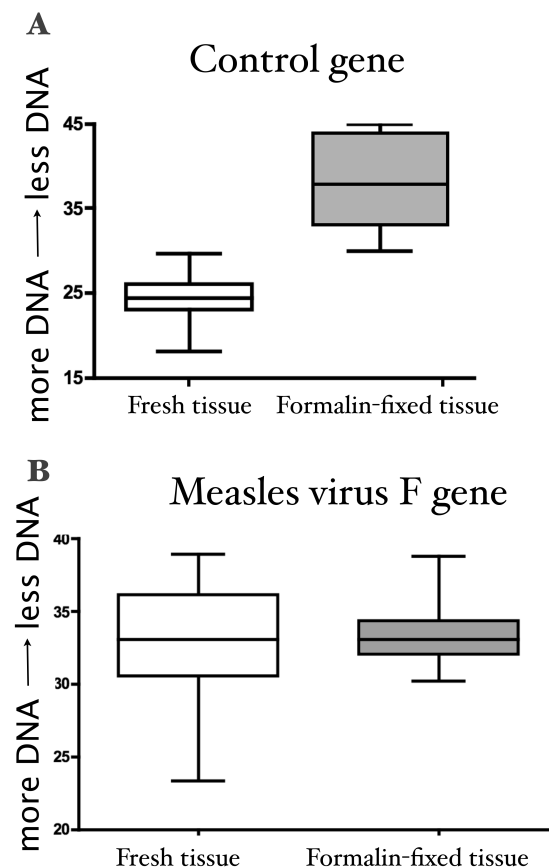


Figure 3. FFPE vs fresh samples. A. Effects of formalin fixation on control gene expression levels showing the difference caused by the formalin fixation process. Due to the exponential nature of the PCR reaction, the difference between the average quantification cycle recorded for fresh tissue and that for formalin-fixed tissue (25 vs 37) equates to 2^{12} or a 4,000-fold reduction of target. B. Absence of an effect of formalin fixation on MeV RNA, with the average quantification cycles very similar.

Since any RNA present during formalin fixation would have been affected in an identical manner, the obvious implication of these results is that whilst the control RNA was indeed present prior to formalin fixation and so was degraded, the MeV target was not degraded and entered the sample after formalin fixation. Consequently, no MeV RNA can have been present in the tissue and the positive result must have been caused by a contaminant.

- b.** Detection of an internal control following RNA extraction is a useful indicator of whether the extraction has been successful: absence of amplification implies that it has not been. Consequently, the O'Leary laboratory standard operating procedures implemented such an assessment and prescribed the exclusion of such samples from further analysis (Figure 4A). Fortuitously from the investigator's point of view and against the rules

of their own SOP, the authors did not discard all samples where the control had been negative. Instead, they reported positive MeV results from samples that contained RNA as well as in autistic patient samples that had been negative for their control (Figure 4B). Since, these samples do not contain RNA by their own definition, their test must be detecting a contaminant.

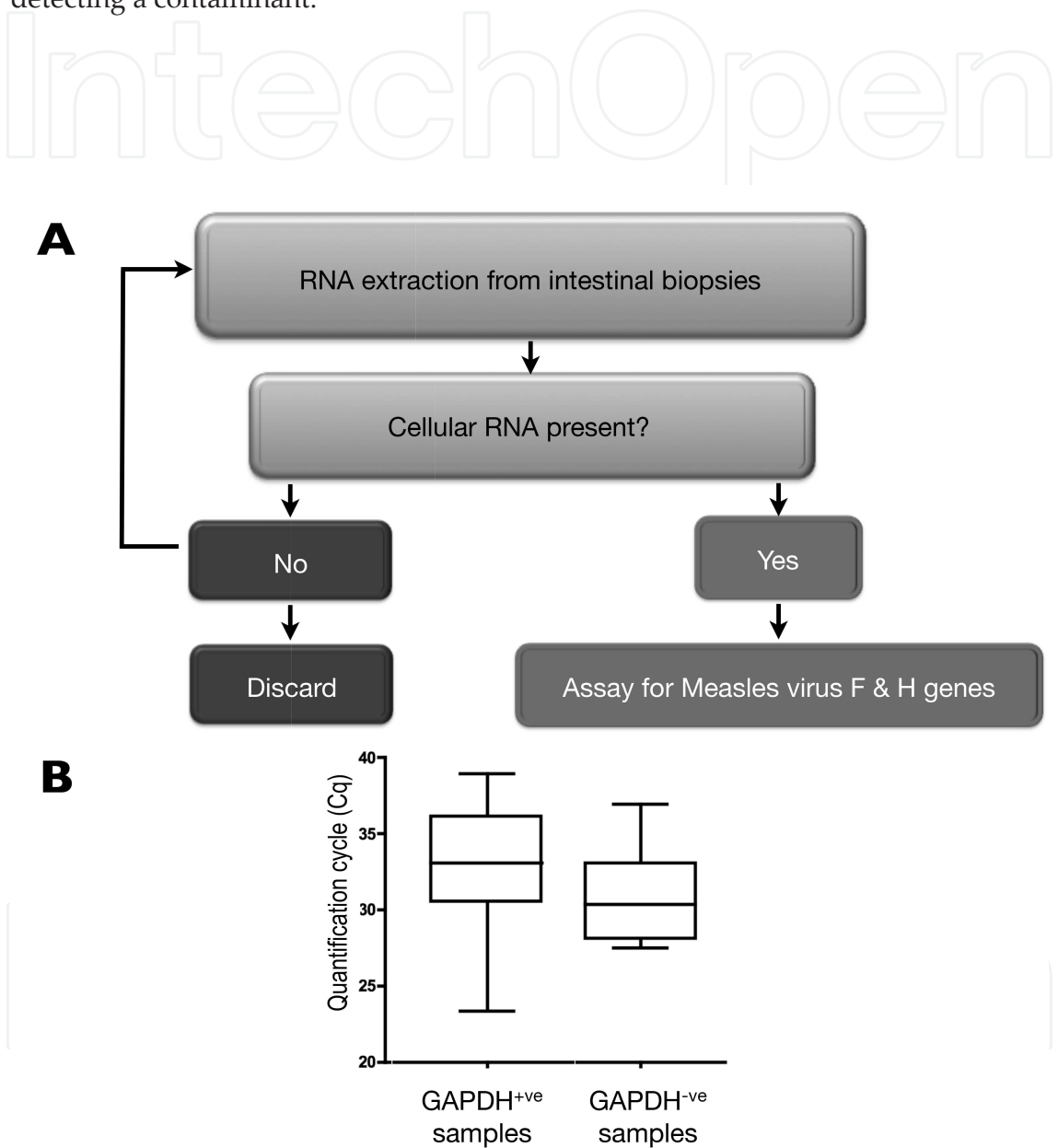


Figure 4. Workflow according to the O’Leary SOP. A. Following RNA extraction, only samples testing positive for GAPDH should have been further analysed for two viral targets. Samples testing negative should have been discarded and fresh RNA extractions attempted. B. MeV target detection from control+ve samples and control-ve samples showing that there is no difference in the quantification cycles.

- c. Two tests accidentally omitted including the RT step before the PCR test. In the case of the control, the results are as expected: the assay works significantly less well (Figure 5A). This is because *Taq* polymerase is very inefficient at making DNA from RNA. In contrast, the four MeV samples tested give the same result, regardless, indicating that the test is detecting DNA (Figure 5B). Since MeV does not exist as DNA, the test is not detecting MeV but a DNA contaminant.

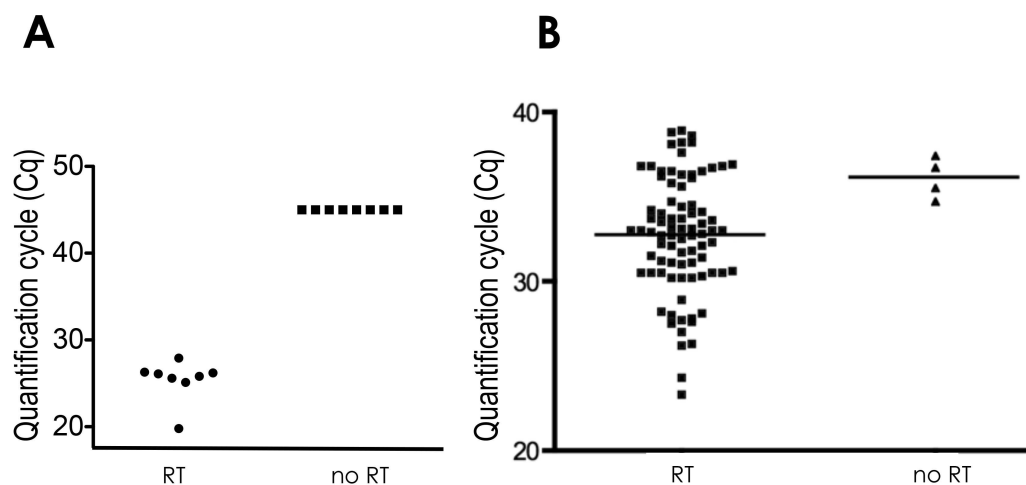


Figure 5. Absence of RT step. A. Control RNA: in the absence of the RT step (no RT), no amplification is observed (a Cq of 40/45 equates by definition to no amplification). B. Measles "RNA": in the absence of the RT step, amplification is observed with Cqs in the same range as in the presence of the RT step (RT).

- d. Successful qPCR amplification is characterised by a characteristic "amplification plot", as obtained from sample F4 in Figure 6A and highlighted by the solid arrow. However a second sample (H2) is also recorded as generating a positive result (circled) even though it is clearly not being amplified (dashed arrow). The correct result is obtained by moving the threshold line (dotted arrow) so that well F4 continues to record a positive results, whereas well H2 now records a Cq of 40, which in this setup equates to "no amplification" (Figure 6B). This is a rather elementary mistake.

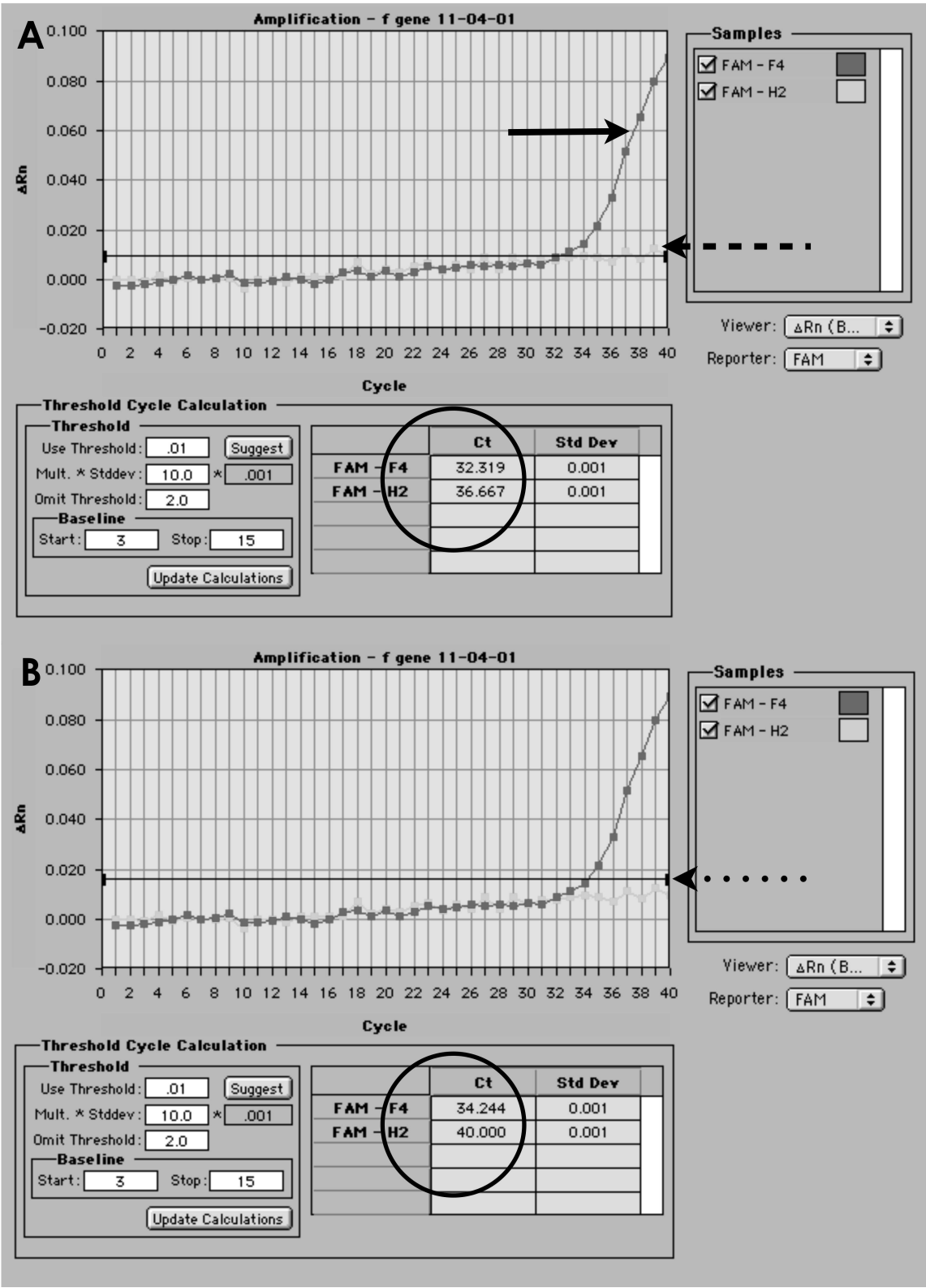


Figure 6. Inappropriate analysis. A. The data as reported by the O’Leary laboratory. B. The re-analysed data showing absence of amplification in well H2 (circled).

3. Interpretation of control results

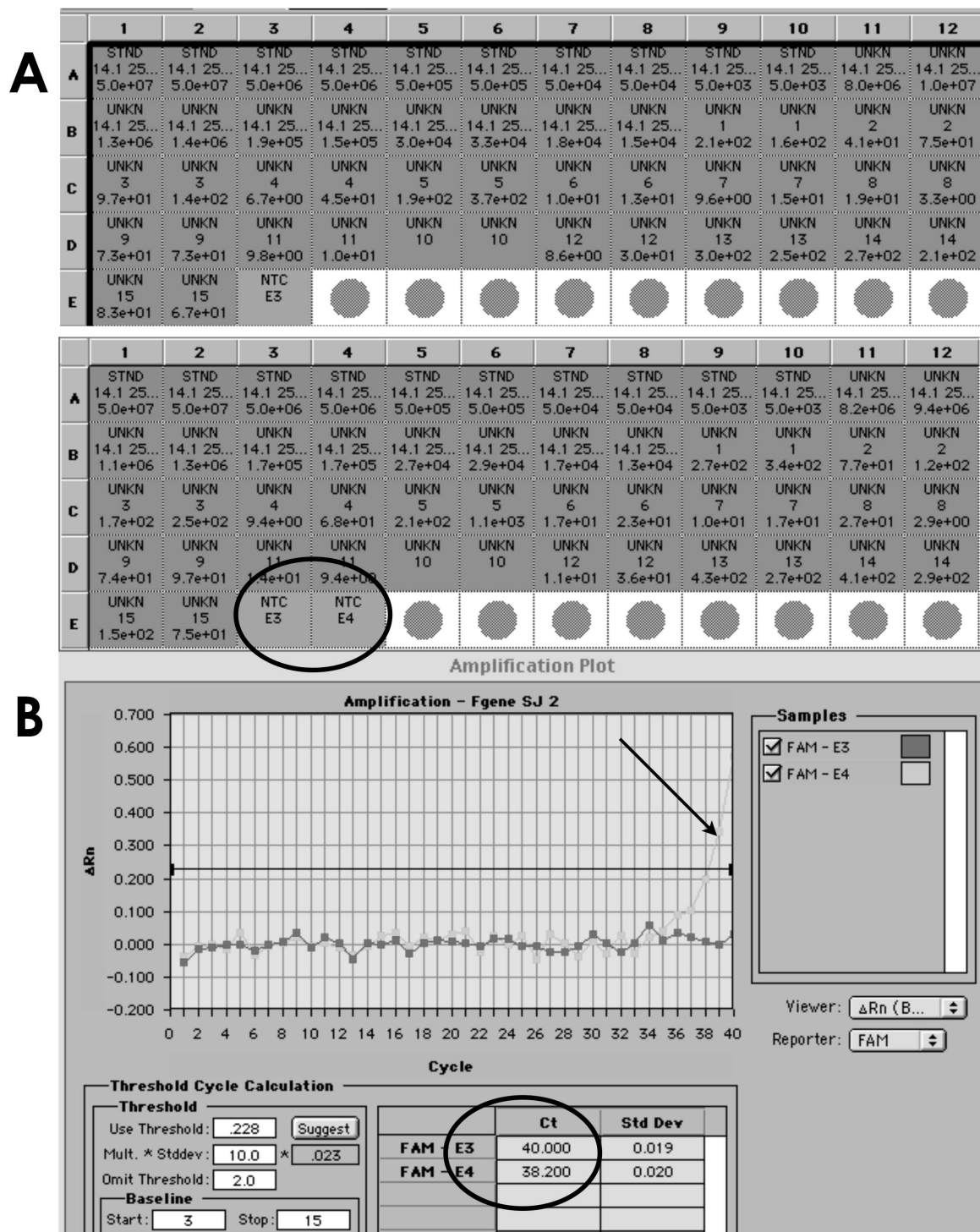


Figure 7. Contamination (1). A. The report submitted by the O'Leary laboratory shows a single negative NTC (circled), with well E4 next to the NTC not analysed, despite all other samples having been analysed in duplicate. B. A re-analysis that includes well E4 shows that this well is contaminated (arrow) and generates a reading (circled), invalidating any results from this run.

- b. Figure 8A shows a similar situation, with all analyses except the NTC in well B11 carried out in duplicate. Again, these were the data disclosed to the investigation, and I had to await the release of the raw data runs to be able to re-analyse those results. This time, a re-analysis of the data including well B12 shows both tests gave positive results, ie both negative controls reported contamination of the test (Figure 8B). The only conclusion from these data can be that the data are unreliable.

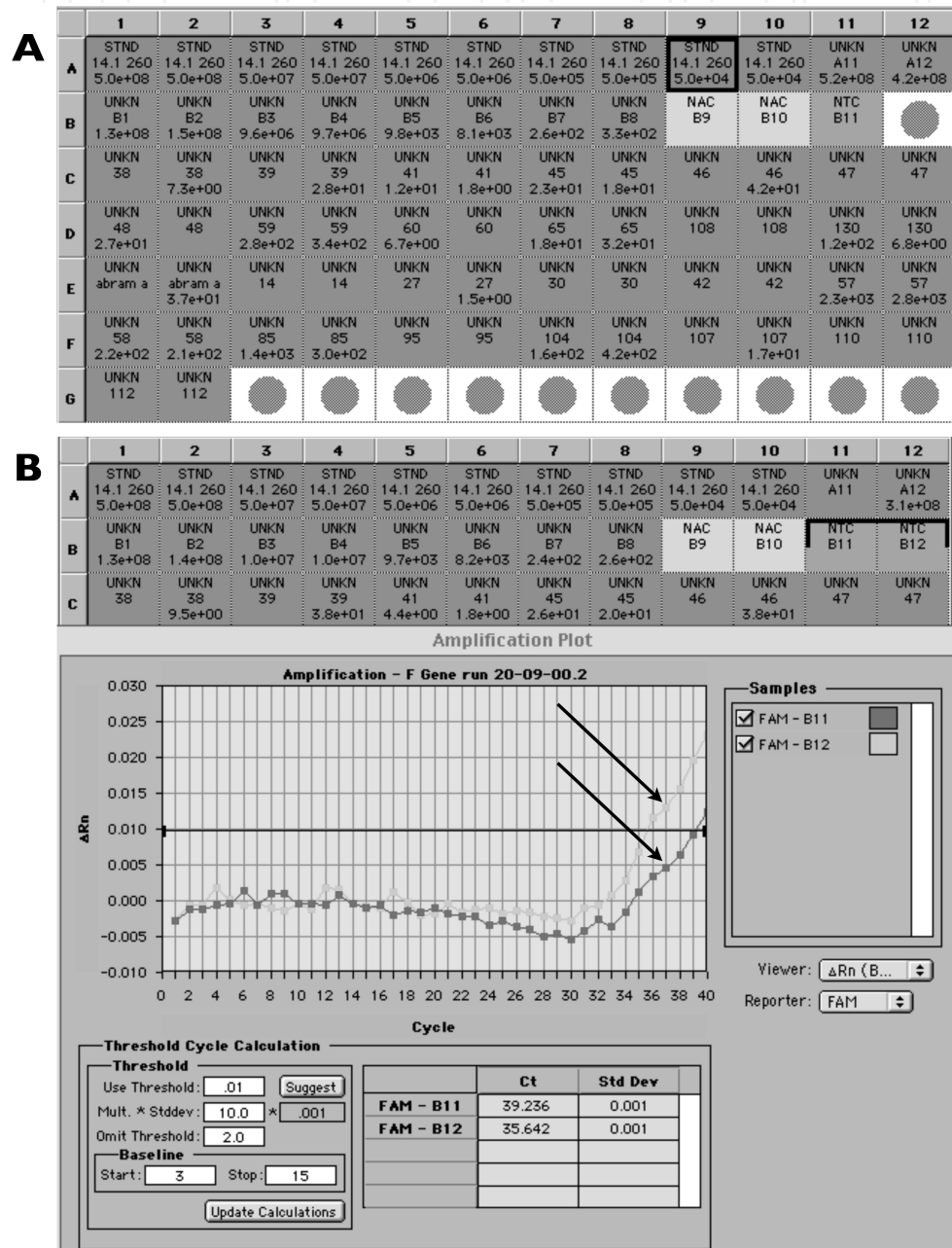


Figure 8. Contamination (2). A. The report submitted by the O'Leary laboratory shows a single negative NTC at B11, with well B12 next to it not analysed, despite all other samples having been analysed in duplicate. B. A re-analysis that includes well B12 shows that both NTCs are contaminated (circled), invalidating any results from this run.

4. Reproducibility of the data

There have been a number of studies attempting to reproduce the findings of the 2002 paper [16-18]. All failed to do so; instead they provided strong evidence for contamination being the cause of the positive findings. However, there were some technical differences between the studies in the choice of tissue (intestine vs blood) or protocols (enzymes, qPCR chemistries). Therefore, whilst there was a strong suggestion that Prof O'Leary's laboratory was detecting contaminants, there was no proof. However, any lingering doubt evaporated with the publication from a multi-centre group of authors that refuted any association between persistent MeV RNA in the gut and autism[19]. Astonishingly, this publication includes the two main authors of the Uhlmann paper, and despite publishing evidence that contradicts their own, they have never retracted their original paper.

5. The MIQE guidelines

The problems inherent in the design and reporting of qPCR-based assays had been known for a long time, but there had been no concerted effort to tackle this serious problem. The 2002 O'Leary paper is not unique in omitting sufficient experimental detail, which impedes the readers' ability to evaluate critically the quality of the results presented, repeat the reported experiments, or integrate methodological advances into their own studies. Indeed, a recent survey of qPCR-based publication demonstrates very clearly that this problem persists even in 2010 [20] and that the perceived quality of the publishing journal is not correlated with the actual quality of the qPCR publication. The egregious use of qPCR in the MeV/MMR/autism context provided the stimulus to initiate a push towards improving the technical aspects of qPCR assay design and reporting. Consequently, guidelines tackling this issue have recently been published which aim to promote consistency between laboratories, and increase experimental transparency [21]. The Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines outline the minimum data set necessary to evaluate effectively RT-qPCR assays, and are designed to be used as a checklist to both accompany manuscript submission and to be available alongside the published manuscript to enhance critical appraisal.

The four key areas of standardisation that define any qPCR experiment are study design, technical detail, analysis methods and statistics. MIQE addresses these under a set of captions that describe a large number of individual elements: "Experimental design, sample, nucleic acids, reverse transcription, target, primers and probes, assay details, PCR cycling and data analysis". At first sight, these look daunting, arduous and over-exacting. In practice, it is clear that most, if not all of these parameters describe information that would be obtained as a matter of course during the experimental design, optimisation and validation stages. Importantly, there is a clear hierarchy with some parameters, labelled "E" (essential) in the published guidelines, indispensable for an adequate description of the qPCR assay, whereas other components, labelled "D" (desirable) more peripheral, yet constituting an effective foundation for the realisation of best practice protocols. There is increasing recogni-

tion that the MIQE guidelines provide the basis for much-needed standardisation as well as encourage the publication of essential information that should be accessible to reviewer and reader[22].

6. Conclusions

This exhaustive analysis of the experimental RT-qPCR data generated by the O'Leary laboratory demonstrates:

- Lack of transparency and completeness of reporting
- Persistent and widespread contamination
- The contamination is caused by DNA
- Inept data analysis

As a result, the conclusions put forward by this paper are entirely incorrect and there is no evidence whatever for the presence either of MeV genomic RNA or mRNA in the GI tracts of any of the patients investigated during the course of the studies reported by O'Leary et al. Instead, it is clear that the data support the opposite conclusion: there is no evidence for any MeV being present in the majority of patients' analysed. Unfortunately, the authors do not report whether any the patients had received the MMR vaccination. However, assuming that a significant proportion had done so, it is also clear that there is no link between the MMR vaccine and the presence of MeV in the intestine of autistic children.

Acknowledgement

This work was carried out for the MMR vaccine litigation trial at the High Court of Justice in London and at the US Vaccine court. The author acted as an expert witness and was paid by the solicitors acting for the principal defendants SmithKline Beecham Plc and Smith Kline & French Laboratories Ltd, Merck & Co Inc and Sanofi Pasteur MSD Ltd. The author also acted as an expert witness for the US Department of Justice and was paid.

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