RT-qPCR and molecular diagnostics: no evidence for measles virus in the GI tract of autistic children

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In 1998 a publication in the Lancet[1] described a pattern of intestinal abnormalities in a group of previously normal children with developmental disorders. It suggested a connection between intestinal and behavioural pathologies and noted that in most cases onset of symptoms was after measles, mumps, and rubella (MMR) immunisation. Although the authors made clear that they had not proven an association between the MMR vaccine and "autistic enterocolitis", they discussed the implication of a causal link between the two. This publication started a major public debate in the UK and elsewhere about a possible link between MMR and autism and resulted in a major crisis of confidence in the safety of the MMR vaccine. Although most of the 13 co-authors later retracted to the interpretation placed upon these findings[2], the debate about a possible link between the measles virus and autism has continued.

In August 2000, a brief, referenced letter signed by JJ O'Leary, V Uhlmann and AJ Wakefield appeared in the Lancet[3]. 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 [referenced] international scientific meetings". Reference 4 refers to a publication by V Uhlmann, O Sheils, K Leittich et al. entitled "Identification of measles virus genomes in ileo-colonic lymphoid hyperplasia in children" as "in press" in the Journal Laboratory Investigations. However, a review of either Pubmed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) or the journal's own website (http://www.nature.com/labinvest/index.html) shows no record of such a publication. The letter also states that "real-time quantitative TaqMan PCR using complementary RNA standards" was used for "quantitation and sequencing of complementary DNA measles virus amplicon" using "three genes N, F, and H of measles virus".

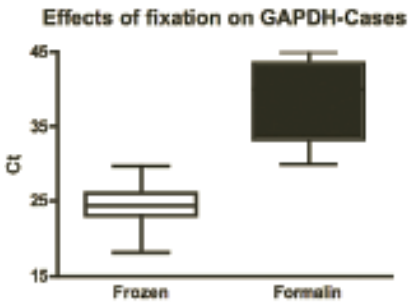

Figure 1: Effects of formalin fixation on control gene. Cts from all fresh/frozen and FFPE samples are shown

In 2002, a paper entitled "Potential viral pathogenic mechanism for new variant inflammatory bowel disease", was published in the journal Molecular Pathology[4]. It describes the use of a RT-qPCR assay to investigate the presence of persistent measles virus in the intestinal tissue of a cohort of children with a "new form of developmental disorder, ileocolonic lymphonodular hyperplasia". The paper claims that 75 of 91 patients with a histologically confirmed diagnosis of ileal lymphonodular hyperplasia and enterocolitis were positive for measles virus in their intestinal tissue compared with five of 70 control patients. It concludes that the data confirms an association between the presence of measles virus and gut pathology in children with developmental disorder.

The reported detection of measles virus in the intestinal tissue of autistic children has been at the centre of contentions of an association between measles virus and autism. It has been used by some to claim a link between the MMR vaccine and the development of a new, regressive form of autism in children. Probably as a direct effect of this controversy, MMR coverage in England fell from a peak of 92% in 1995 to 80% in 2003/04, and even to-day the latest statistics show an uptake rate of only 84% in England and Wales, well below the 95% required for herd immunity (http://www.ic.nhs.uk/statistics-and-data-collections/health-and-lifestyles/immunisation/nhs-immunisation-statistics-england-2007-08-%5Bns%5D) .

"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"

In London, vaccine uptake was as low as 70% in 2003/04 and even by 2006 stood at only 73% for the first dose at age two (2006 data from 22/31 primary care trusts). Coverage in some areas, e.g. Kensington and Chelsea, was as low as 52% with several others in the high fifties to low sixties. In Greenwich, only 61% and 33% were vaccinated by their second and fifth birthdays,

respectively. 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.

There have been a number of studies attempting to reproduce the findings of the paper originating from JJ O'Leary's laboratory[5-7]. All failed to do so; instead they provided strong evidence for contamination being the cause of the positive findings reported by Ulmann et al. However, there were some technical differences between the original study and the three more recent ones in the choice of tissue (gut vs. blood) or protocols (enzymes, real-time PCR

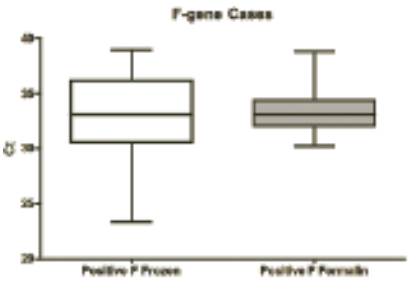

Figure 2: Effects of formalin fixation on F-gene. Cts from samples that tested positive for F-gene are shown for fresh/frozen and FFPE

chemistries). Therefore, whilst there was a strong suggestion that Prof O'Leary's laboratory was detecting contaminants, there was no proof. This has now changed with a recent publication, which includes Prof O'Leary and Dr Sheils, that has used the same methods, *inter alia*, as those originally published, to come to the conclusion that there is indeed no link between measles virus vaccine, autism and enteropathy[8]. Astonishingly, there is no attempt to retract the original report, and the admission that these results are in direct contradiction of the previously reported ones are thoroughly disingenuous: "Our results differ with [their own] reports noting MV RNA in ileal biopsies of 75% of ASD vs. 6% of control children [10,41]. Discrepancies are unlikely to represent differences in experimental technique because similar primer and probe sequences, cycling conditions and instruments were employed in this and earlier reports; furthermore, one of the three laboratories participating in this study performed the assays described in earlier reports. Other factors to consider include differences in patient age, sex, origin (Europe vs. North America), GI disease, recency of MMR vaccine administration at time of biopsy, and methods for confirming neuropsychiatric status in cases and controls."

On 11 June 2007, the first of approximately 4,800 cases in the US omnibus autism proceedings came to trial at the United States Court of Federal Claims, where all vaccine claims are managed and adjudicated by the congressionally created Office of Special Masters. The trials are designed to establish whether or not autism can be caused by thimerosal containing vaccines, by MMR vaccine, or a combination of the two.

On 6 June, the UK High Court gave permission for the release to the US Secretary of the Department of Health and Human Services of two reports authored by the author (Stephen Bustin). These documents were filed by the three principal defendants in the UK MMR vaccine litigation. They constitute an exhaustive analysis of raw TaqMan data underlying the results reported by the Uhlmann paper that were generated at the laboratory of Unigenetics Ltd at Coombe Women's Hospital in Dublin by Professor O'Leary's team, which included Dr Sheils.

On 20 June, the evidence contained in these reports was presented to the trial, and this communication outlines the major conclusions and is based upon the publicly available transcript from the autism omnibus proceedings (ftp://autism.uscfc.uscourts.gov/autism/transcripts/day08.pdf).

Real-time RT-PCR

The RT-qPCR assay consists of a reverse transcription step that converts RNA into DNA, followed by the real-time PCR segment, which specifically amplifies the target gene(s) of interest. The use of fluorescent reporter molecules permits concurrent target amplification, detection and quantification as the assay proceeds[9,10]. Increases in fluorescent signal are proportional to the amount of DNA produced during each PCR cycle and produce characteristic threshold cycles (C_t) for every reaction. The C_t is defined as the PCR cycle fraction at which the signal first rises above instrument background fluorescence. As a result, the more initial targets, the sooner the instrument can detect the fluorescence and the lower the C_t. Conversely, a higher C_t 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[11]. In practice, the quality of any data obtained using the RT-qPCR assay is affected by the integrity of the RNA template, the choice of cDNA priming strategy and reverse transcriptase, the characteristics of the primers and the validity of the normalisation method[12-18]. Not surprisingly, the universal dissemination of this technology has led to the development of numerous, individual experimental protocols that can affect data

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reproducibility[19,20]. The resulting potential for uncertainty has increased the awareness of a need for common guidelines, in particular those relating to quality assessment of every component of the RT-qPCR assay and appropriate data analysis[11]. This clear requirement for improved consistency of gene expression measurements is particularly relevant in relation to human clinical diagnostic assays[21-23].

Analysis of the published "Uhlmann" paper

The results presented in this paper[4] are at the core of most hypotheses linking measles virus and autism. Consequently it is essential to consider the main criticisms of the published evidence:

1. Total RNA was extracted from fresh frozen biopsies as well as formalin fixed paraffin-embedded tissue (FFPE). Formalin fixation is used to preserve tissue morphology without regard for RNA preservation. It is well established that this process modifies and destroys RNA, or in Prof O'Leary's own evidence "wax and fixation by itself breaks down RNA"[24]. This makes it less accessible for amplification compared with RNA from fresh samples and results in an increased

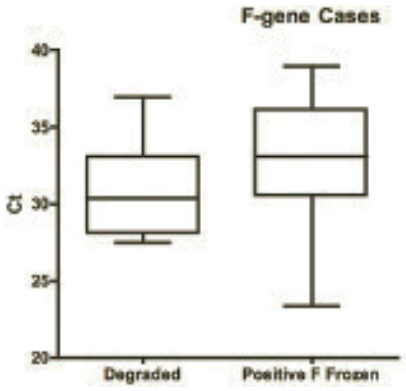

Figure 3: Comparison of F-gene Cts from degraded and non-degraded RNA

Ct for FFPE samples that is variable and assay-dependent[25]. The significant qualitative differences between the RNAs make it inappropriate to compare directly any RT-qPCR results[26]. Since the Uhlmann paper provides no information as to whether the same proportion of fresh/frozen and FFPE samples were used for patients and controls, it is not possible to gauge the reliability of the RT-qPCR data.

2. There is no information as to how the fresh samples were frozen or how long they had been stored. No quantification or quality assessment of the extracted RNA is provided; indeed there is no mention of RNA quality. This is vital information needed to assess the validity of any quantitative or negative result[21].
3. A quantitative comparison of RT-qPCR data from different individuals requires the inclusion of a gene dosage compensation (or "normalisation") step. This involves the separate amplification and quantification of internal reference (or "housekeeping") genes, and expressing gene-of-interest copy numbers relative to those of the reference genes. The authors mention gene dosage correction, but give no indication of what they actually did. Furthermore, they used a single gene only, GAPDH, as their reference gene. Normalisation using a single reference gene, and GAPDH in particular, is not reliable due to the significant variation in its expression between and even within individuals[27]. Since the performance of the RT-PCR assay is crucial to accurate quantification, information with respect to the GAPDH assay should have been provided.
4. As with any biochemical assay, RT-qPCR experiments use standards that permit the calculation of target RNA copy numbers, PCR amplification efficiencies and assay sensitivity and linearity. Ideally they should be included with every assay[12,13]. The paper mentions standards, but does not provide any of the crucial information about what their use revealed with respect to the quality of the RT-qPCR assay.
5. Although the paper describes a number of controls, it omits the elementary one of leaving out the RT-step and adding the RNA directly to a PCR assay. This is required to discriminate amplification of RNA from that of a DNA target: if positive, the target must be a contaminating DNA. Since MV has no DNA stage in its life cycle, inclusion of this control is absolutely essential. As the assay uses *Tth* polymerase, this control should have consisted of either leaving out the RT step or using *Taq* polymerase, which is a poor RNA-dependent DNA-polymerase.

"Since MV has no DNA stage in its life cycle, inclusion of this control is absolutely essential"

6. The TaqMan results are summarised without providing any actual data. Simply stating 70/91 positive in group A and 4/70 in group B is meaningless. The whole point of a TaqMan assay is to provide quantitative data. The TaqMan methods refer to MV F, and H genes, but there is no mention of any concordance in the results section of the paper. If genuine, results from all three genes should have been similar. Since the abstract claims to have quantitated both F and H genes, it is unusual that the results were not included.
7. MV copy numbers in the affected children are reported as ranging from 1 to $3x10^5$ copies of RNA/ng total RNA; no corresponding figure is provided for the 4/26 positive samples from the control samples. In addition, there is no information on how that copy number was obtained and on whether replicates were run and gave concordant results.

One purpose of reporting results in the scientific literature is to provide sufficient information to allow any competent scientist to repeat that experiment. This requires detailed publication of the protocol. In addition, any conclusions must be supported by data and it is unacceptable to summarise results without any relevant information as to how those were obtained. At the very minimum, standard curve data and copy number data for F and H genes should have been published. The standard curve data would allow the reader to assess the quality of the assay, and concordance between viral F and H gene results would make the data more convincing, since they would act as independent internal controls of one another.

Analysis of the raw TaqMan data underlying the "Uhlmann" paper

The transcript of the detailed criticisms of the work underlying this paper is available online (ftp://autism.uscfc.uscourts.gov/autism/transcripts/day08.pdf) and addresses all of the above points. The purpose of this communication is to highlight the two key lines of reasoning that prove with the highest degree of certainty that Prof O'Leary's laboratory was detecting contaminants, and that these contaminants were DNA. This is a decisive finding, since MV does not exist in DNA form. Hence the assay cannot be detecting MV and, in the absence of this bedrock evidence, any proposed link between measles virus, MMR and autism is demonstrably unsustainable.

This conclusion was reached after examining the data from every single sample from all 91 RT-qPCR runs disclosed by Unigenetics. This involved using identical sequence detection software (SDS) and versions thereof, to process the disclosed

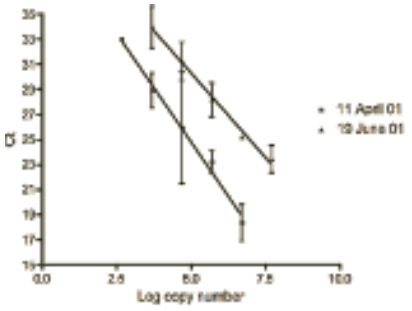

Figure 4: F-gene standard curves. On 11/04/01 the RT step was included, on 19/06/01 it was omitted

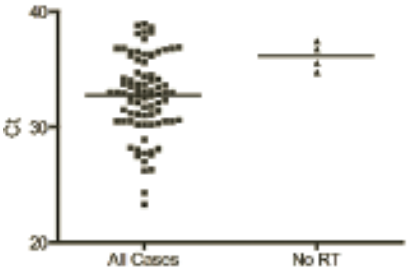

Figure 5: Similar Cts with and without reverse transcription. Comparative Cts for the F-gene from RNA subjected to an RT step, followed by a PCR (all cases) and RNA subjected only to a PCR step (no RT)

TaqMan files and generate new experimental reports. These were compared with those disclosed by Prof O'Leary's 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 instrument. This involved an examination of nearly 700 data points per well and permitted a definitive resolution of all ambiguities.

I also read the disclosed experimental reports, operator sheets and laboratory notebook entries relevant to the RT-qPCR assay.

I compared these with the data obtained from every run. I examined the standard operating procedure and noted where the different operators did not adhere to the set protocols.

"Uhlmann" is detecting contamination

RNA was extracted from both fresh/frozen and FFPE tissue samples and subjected to RT-qPCR analysis of a reference gene, GAPDH, as well as the MV F-gene. The expectation was that the average C_t for both would be significantly higher in the FFPE samples. Indeed, for GAPDH mRNA there was an average difference of just over nine C_ts between fresh/frozen and FFPE samples, corresponding to an approximate 500-fold difference in mRNA levels.

"Quantification of an internal reference mRNA is useful as a rough guide to RNA quality"

This result provided a convenient control for the MV F-gene assay, with the expectation of a similar ΔC_t between the two sample types. Unexpectedly, the F-gene C_ts recorded for RNA extracted from fresh/frozen and FFPE samples were approximately the same.

Since any nucleic acid present during formalin fixation would have been affected in an identical manner, the obvious implication of these results is that the GAPDH target was present prior to formalin fixation, whereas the F-gene target entered the sample after formalin fixation. Consequently, the F-gene assay is detecting a contaminant. The finding of F-gene amplification in approximately one third of F-gene no template (negative) controls supports this conclusion.

The contaminant is DNA (1)

Quantification of an internal reference mRNA is useful as a rough guide to RNA quality: absence of amplification suggests degradation or lack of RNA and allows exclusion of such samples from further analysis. Again, the expectation was that any GAPDH -ve sample would also be negative for the MV F-gene. Fortuitously from the investigator's point of view and against the guidelines of their own SOP, the authors did not discard all GAPDH -ve samples. Instead, they reported positive MV F-gene results in several GAPDH -ve autistic patient samples. Implausibly, the average C_t recorded for the F-gene from these samples was 3 C_ts lower than that recorded

for GAPDH +ve samples, implying an eight-fold higher abundance of F-gene target (see Figure 2 on page 12). Since, by definition, these samples do not contain amplifiable RNA, the PCR must be detecting contaminating DNA.

The contaminant is DNA (2)

The inference that the O'Leary assay is detecting DNA is backed by the chance discovery that two RT-PCR runs, one for the GAPDH gene the other for the MV F-gene, have accidentally omitted the reverse transcription step. Ironically, this constitutes the negative control referred to above and, if positive, proves that the amplified target is DNA, not RNA. Prof O'Leary used cRNA to generate standard curves for both the GAPDH and MV F-gene assays. A comparison of two F-gene standard curve runs, one carried out in the presence of the RT step (11/04/01), the other in its absence (19/06/01), clearly shows the effect of the omitted RT.

The ΔC_t is around eight, corresponding to an approximate 250-fold difference in apparent abundance; the results from the RT-qPCR assay targeting GAPDH are similar. These results are as expected: omission of the RT-step results in inefficient reverse transcription of the GAPDH and MV F-gene cRNA standards, as well as GAPDH mRNA.

"These data provide incontrovertible evidence that the target detected by Prof O'Leary's laboratory is DNA, not RNA"

Opportunely, the F-gene run of 19/06/01 contained four autistic patient samples. All four recorded positive C_ts that, against expectation, were in a similar range to most of the F-gene C_ts recorded from runs that included the RT step.

These data provide incontrovertible evidence that the target detected by Prof O'Leary's laboratory is DNA, not RNA. Since MV does not pass through a DNA stage during its life cycle, the assay cannot be detecting MV.

Conclusion

This exhaustive analysis of the experimental RT-qPCR data generated by the Unigenetics laboratory demonstrates persistent and widespread contamination with F-gene DNA. As a result there is no credible evidence for the presence of either MV genomic RNA or mRNA in the GI tracts (or blood samples) of any patient investigated by this laboratory. Consequently, this finding excludes any link between MV and, by extension, the MMR vaccine and autism.

"Opportunely, the F-gene run of 19/06/01 contained four autistic patient samples"

Acknowledgement

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Stephen Bustin obtained his PhD from Trinity College, University of Dublin in molecular genetics. Since 1989 he has worked at the Royal London Hospital, aiming to apply his research in a more direct, practical setting. Following promotion to Senior Lecturer (1995) and Reader in Molecular Medicine (2002) he was awarded a personal chair by the University of London in 2004. He was appointed as a visiting Professor of Molecular Biology by the University of Middlesex in 2006.

His main area of research is into bowel-associated pathologies, especially colorectal cancer and, more recently, Clostridium difficile-associated disease. He has a special interest in molecular technologies and his laboratory operates at the forefront of technological development in nucleic acid quantification, where he is an internationally acknowledged leader. He has given numerous presentations at scientific conferences around the world. He has organised and co-organised many qPCR meetings in the UK, Europe and the US.