Respondent's Exhibit UU

Critique of Dr Karin D Hepner's letter, dated May 21 2007, concerning the plausibility of an MMR-ASD causality hypothesis.

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Summary

This report deals with the first question posed by Dr Hepner, which concerns the validity of the RT-PCR study carried out in Prof O'Leary's laboratory. This question is entirely appropriate and, if dealt with properly, should address the issue of whether that study is reliable and scientifically sound. However, the discussion of that question, although specious, is ill-informed, inadequate and fallacious. Dr Hepner does not address any of the shortcomings of Prof O'Leary's publication, and fails to apply any of her (correct) quality measures to that body of work. Her criticism of work designed to reproduce Prof O'Leary's work is tendentious and largely unconvincing.

1. Question addressed in this report

Dr Hepner asks whether "*the studies which demonstrate the presence of MV-RNA in ASD-GI biopsy [are] reliable and scientifically sound?*". She answers by analysing "*the data and methods of the "Uhlmann paper*" …[and] *then examine[s] the published criticisms of these studies*".

Dr Hepner correctly describes several criteria that need to be examined for "*determining the efficacy of a scientific study:*

I-Controls and cross-contamination: Accuracy of results and interpretation of data depend on the use of controls. Were proper positive and negative controls used? Does the standard operating procedure limit the probability of cross-contamination?

II-Experimental design: Were the assays used in the Uhlmann study appropriately selected and implemented?

III-Consistency and reproducibility: Results using different assays within a single study should be concordant, and should also be reproducible in other laboratory settings. Are the results from this study internally consistent and reproducible?"

2. Experimental controls and cross-contamination

Dr Hepner divides controls into "*two subcategories:*

A-those that can detect flaws in the experimental design, e.g. a "positive" control and a "negative" control, both of which must act consistently true to designation, and
B- those that monitor for the presence of MV RNA in developmentally normal GI controls (simultaneously with ASD-GI samples), in order to draw conclusions about the significance of the presence of MV RNA".

Put slightly differently, A refers to technical controls that delineate the veracity and validity of the RT-PCR assay and any conclusions reached from its results. B, on the other hand, refers to biological controls, whose results are important for the interpretation of any RT-PCR data, but which depend entirely on the validity of the data obtained from "A". It is essential to understand that "B" depends entirely on "A" and unless the assay itself is completely transparent, convincing and verifiable, no conclusion can be reached with regards to any data.

Dr Hepner's discussion of positive and negative controls (page 2) is entirely reasonable until she states that: "*This list of controls is undoubtedly sufficient. … In the Uhlmann study, both positive and negative controls were properly selected and acted true to designation*".

It is impossible to ascertain whether the controls "*acted true to designation*" for the TaqMan assay. The authors only refer to a negative technical (no-template) control in the context of their conventional RT-PCR assays in the "Results" section (below).

> The specificity of the primer/probe sets to detect MV F and H genes was established using RNA extracted from: (1) cryo-preserved ileal biopsy material from four affected children, (2) SSPE brain, and (3) MV infected Vero cells (fig 2A). All four patient samples were positive for MV F and H genes by TaqMan RT-PCR. Amplicon specificity was confirmed by Southern blot analysis using F and H gene specific probes (fig 2B,C). No template controls run in parallel were negative.

No mention of negative controls is made when describing the actual experimental results for the TaqMan assay from patient samples. This is in contrast to the *in situ* RT-PCR data, which refer to negative results from negative controls (see below).

> using RT in situ PCR (fig 4A). Signal was not detected in similarly processed normal brain, or when irrelevant PCR primers were used on sections of SSPE brain (fig 4B).

The reader has to assume that O'Leary included no-template controls with his actual samples and that these were negative. Therefore it is surprising that Dr Hepner concludes that "*negative controls were properly selected and acted true to designation*", when there is not only no information about whether these controls (principally the no template controls) were included, but, assuming that they were, about how they were handled: in duplicate, were tubes sealed before and after dispensing of reagents, where were the controls located relative to samples, were they always concordantly negative?. In any case, negative controls cannot guarantee against sporadic contamination, although a sporadic contamination event is unlikely to occur twice in exactly the same way.

Dr Hepner discusses the precautions taken to prevent contamination. In principle these were the correct measures and are discussed appropriately. However, no mention is made of how TaqMan reaction plates are disposed of after the assay; this is important as the end of the assay is the most likely chance of laboratory contamination from the amplification products generated from positive controls and standards. Furthermore, the experience with clinical specimens at NIBSC [1] and at the Royal Free Hospital [2] has shown that even with the most meticulous technique, cross contamination of specimens can occasionally occur. The problems with contamination are also addressed by the D'Souza paper [3].

Dr Hepner states that "*controls confirmed that they were successful*". As discussed above, no actual mention of no-template controls is made in the context of assaying patient samples.

Dr Hepner discusses the use of "*controls that serve as a baseline for the presence or absence of the gene under examination*", i.e. biological controls. She points out that O'Leary's control samples were derived from GI biopsies, whereas the

most recent Afzal and D'Souza papers do not use GI tissue. Consequently, she maintains that "*This is comparing apples to oranges. When one is dealing with a low copy target and an experimental strategy already hovering around its detection threshold, one may not compare tissue samples from different sources, or draw conclusions about the presence of MV RNA in GI tissue based on what is seen in PBMCs or other tissues. Therefore, it is important to note that any study which has attempted to refute the findings of the Uhlmann study by using an RNA source derived from anything other than GI tissue has violated a basic principle of proper control tissue selection*".

It is true that ideally one would choose the same controls as those used by the authors of an experimental protocol one wishes to reproduce. Dr Hepner fails to mention that several laboratories did attempt to find MV in GI biopsies from patients with inflammatory bowel disease using RT-PCR technology. Although one study was able to identify a measles virus genome sequence in the positive technical control samples that had measles virus corresponding to as little as $5.5x10^{-3}$ plaque forming units, no tissue sample from IBD patients derived from endoscopic biopsies was positive for measles specific nucleic acid [1]. In another study, RT-nested PCR assays specific for the N and F genes were reported to have a sensitivity capable of detecting a single genome copy, but again no MV was found in tissue biopsies [4]. Similar results were reported by other studies [5,6].

Dr Hepner's conclusion that "*in regard to controls, this work was performed in a rigorous manner*" is not sustainable, as there is simply not sufficient information provided in this publication.

3. Experimental Design

Dr Hepner writes that *"the O'Leary lab used … a version of classic PCR that allows for increased assay sensitivity due to exponential amplification of the target gene, known as TaqMan PCR*". The O'Leary lab is of course the laboratory where Dr Uhlmann worked and where the work described in the Uhlmann paper was carried out. Every PCR reaction amplifies target exponentially. The difference with TaqMan is that the assay uses a fluorescent probe to detect the amplification products in real-time.

Dr Hepner states that "*concordant data was generated*" and "*The studies conducted in John O'Leary's laboratory stand up to basic scientific criticism. Proper controls were employed*". The paper presents only outline details of protocols employed and, critically, does not report any TaqMan data, only summary of results. Consequently, it is impossible to tell whether the data were concordant. Furthermore, the studies can be (and have been) subjected to basic scientific criticism and, as discussed earlier, it is not known whether proper controls were employed.

At this stage it is worth looking at the experimental design to determine whether the study was carried out in a technically correct and properly controlled manner.

3.3. RNA

Since the target of this assay is RNA, a highly labile nucleic acid, it is worth investigating what the paper has to report about sample preparation and RNA quality assessment, two critical parameters for generating reproducible data [7].

Regrettably, there is no mention of how samples are handled or whether RNA quality was assessed in any way. Furthermore, some samples were obtained from fresh/frozen biopsies and others from formalin-fixed samples (see below). The "Materials and Methods" section provides no information on how many biopsies were fresh/frozen or formalin-fixed or whether the numbers were equal for patients and controls. This is essential information, as different results would be expected for samples extracted from fresh biopsies compared to those obtained following formalin fixation. There is no further mention of this, and constitutes a fatal flaw for any conclusion based on the TaqMan data obtained from these biopsies. Dr Hepner does not mention this.

MATERIALS AND METHODS

Patients and RNA extraction

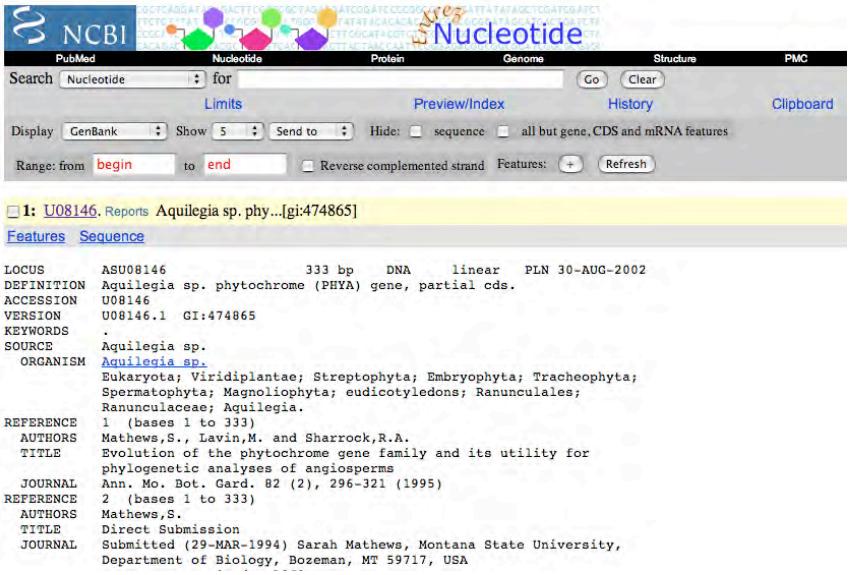
All patient samples were provided by the department of gastroenterology, Royal Free Hospital, London, UK. Ileal lymphoid tissues from 91 affected children were examined (median age, 7 years; range, 3–14; 77 boys). Developmentally normal paediatric controls (n = 70; range, 0–17 years; 47 boys) included: 19 children with normal ileal biopsies, 13 children with mild non-specific chronic inflammatory changes, three children with ileal lymphonodular hyperplasia (LNH) investigated for abdominal pain, eight children with Crohn's disease, one child with ulcerative colitis, and 26 children who had undergone appendicectomy for abdominal pain including appendicitis.

MV positive control material included two cases of SSPE and MV infected Vero cells. Negative control material included uninfected Vero cells, and human tissues, control RNA extracted from Raji cells (Applied Biosystems, Foster City, California, USA) and normal peripheral blood mononuclear cells.

Total RNA was extracted from fresh frozen biopsies, peripheral blood mononuclear cells, and MV infected and uninfected Vero cell lines using the Ultraspec-11 RNA isolation system (Biotecx Laboratories, Houston, Texas, USA). Total RNA was extracted from formalin fixed, paraffin wax embedded tissues using the Purescript® RNA isolation kit (Gentra Systems, Minneapolis, Minnesota, USA).

3.4. Probe

The O'Leary paper states that "measles virus F-gene primers and probes were designed based on the following eleven PubMed sequence entries: X16565, U03655, U03666, U03648, U03662, U08146, U03657, U03651, U03659, AJ133108, X16567"and lists the F-gene probe sequence in Table 1.
U08146 is not a measles virus sequence, as is evident from this extract from the relevant sequence entry in PubMed:



This suggests a certain carelessness in the compilation of the paper.

A sequence comparison of the remaining ten of the sequences referred to in Table 1 of the "Uhlmann" paper reveals the following consensus for the sequence targeted by the probe:

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AJ133108   CTGCACGAGGGTAGAGATTGCAGAATACAG
U03648     CTGCACGAGGGTAGAGATTGCAGAATACAG
U03651     CTGCACGAGGGTAGAGATTGCAGAATACAG
U03655     CTGCACGAGGGTAGAGATTGCAGAATACAG
U03657     CTGCACGAGGGTAGAGATTGCAGAATACAG
U03659     CTGCACGAGGGTAGAGATTGCAGAATACAG
U03662     CTGCACGAGGGTAGAGATTGCAGAATACAG
U03666     CTGCACGAGGGTAGAGATTGCAGAATACAG
x16567     CTGCACGAGGGTAGAGATTGCAGAATACAG
x16565     CTGCACGAGGGTAGAGATTGCAGAATACAG

Consensus CTGCACGAGGGTAGAGATTGCAGAATACAG
```

All sequences have a T at position 19 (highlighted in red). However, this is not the sequence reported in the paper, as shown below:

Table 1 Measles virus primer and probe sequences

Primer/Probe	Sequence 5′–3′	Amplicon size
N1 forward	5′ TCA GTA GAG CGG TTG GAC CC 3′	
N1 reverse	5′ GGC CCG GTT TCT CTG TAG CT 3′	150 bp
N2 forward	5′ GAG TCG AGG AGA AGC CAG GG 3′	
N2 reverse	5′ GCT GGA CTC CGA TGC AGT GT 3′	120 bp
H1 forward	5′ TTC ATC GGG CAG CCA TCT AC 3′	
H1 reverse	5′ CTC TGA GGT GTC CTC AGG CC 3′	150 bp
H2 forward	5′ TGG GCA CCA TTG AAG GAT AA 3′	
H2 reverse	5′ AAC CGT GTG TGA TCA ATG GC 3′	120 bp
F1 forward	5′ TGA CTC GTT CCA GCC ATC AA 3′	
F1 reverse	5′ TGG GTC ATT GCA TTA AGT GCA 3′	150 bp
F2 forward	5′ CCC ACC GGT CAA ATC CAT T 3′	
F2 reverse	5′ CCC TCG TGC AGT TAT TGA GGA 3′	150 bp
GAPDH 1	5′ GAA GGT GAA GGT CGG AGT 3′	
GAPDH 2	5′ GAA GAT GGT GAT GGG ATT TC 3′	226 bp
N1 probe	5′ CAA ACA GAG TCG AGG AGA AGC CAG GGA 3′	
H1 probe	5′ CCG CAG AGA TCC ATA AAA GCC TCA GCA C 3′	
F1 probe	5′ CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG 3′	
GAPDH probe	5′ CCG ACT CTT GCC CTT CGA AC 3′	

Measles virus N gene primers and probes were designed based on the following Genbank sequence entries (accession numbers): X16565, S58435, NC_002494, NC_002496, X01999, U03661, U03658, and U03656. Measles virus H gene primers and probes were designed based on the following GenBank sequence entries: X16565, U03649, U03654, U03669, U03660, U03671, U03667, Z80793, AF045204, Z66517, and Z80816. Measles virus F-gene primers and probes were designed based on the following Genbank sequence entries: X16565, U03655, U03666, U03648, U03662, U08146, U03657, U03651, U03659, AJ133108, X16567.

When aligned, the sequences read as follows:

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                CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG
                *** *** *** *** *** ***  ** *** *** ***
Consensus       CTG CAC GAG GGT AGA GAT TGC AGA ATA CAG
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This means that the probe has a single base mismatch with the measles virus sequences from Table 1 used by O'Leary to design the F-gene probe and suggests lack of care on the part of the researchers. Checking the sequences of primers and probes should be a very basic examination before any assay is run. The probe is of critical importance to the success of a TaqMan assay because it is a key determinant of the assay's specificity. Its nucleotide sequence is complementary to its target, here the MV F-gene, and if the sequence is not 100% complementary, there can be problems with assay sensitivity and reproducibility. In practice, this probe will detect F-gene target, indeed it may be that there is some sequence variability around this region, but the mismatch may compromise the specificity, sensitivity and reproducibility of the assay. However, without detailed comparisons between correct and mismatched probes, and without further investigation of the actual sequence around that region of the MV genome it is impossible to resolve the actual effects of using this incorrect probe. Importantly, the fact that an incorrect probe was used suggest that Dr Hepner's comments that "*The studies conducted in John O'Leary's laboratory stand up to basic scientific criticism*" and that his studies were "*technically accurate*" cannot be sustained.

3.5. MV Targets

The primer detail presented in Table 1, and the contents of the "Materials and Methods" and "Results" sections of the paper indicate that the investigators are targeting the MV F and H genes in the TaqMan assays.

A gene dosage correction was carried out using glyceraldehyde phosphate dehydrogenase as a housekeeping gene. Measles virus quantitative TaqMan RT-PCR was performed by generating standard curves for the F and H genes. Taqman RT-PCR standards were generated by cloning the F and H gene specific PCR products into a vector using the TOPO TA cloning® system

RESULTS

Overall, 75 of 91 affected children had MV RNA in their ileal lymphoid tissue compared with five of 70 in the control patient cohort (Fisher exact test, $p < 0.0001$; Analyse-it Software, General 1.62).

A total of six different PCR primer sets were optimised by solution phase RT-PCR to amplify the MV F, H, and N genes from RNA extracted from MV infected Vero cells (fig 1).

The specificity of the primer/probe sets to detect MV F and H genes was established using RNA extracted from: (1) cryopreserved ileal biopsy material from four affected children, (2) SSPE brain, and (3) MV infected Vero cells (fig 2A). All four patient samples were positive for MV F and H genes by TaqMan RT-PCR. Amplicon specificity was confirmed by Southern blot analysis using F and H gene specific probes (fig 2B,C). No template controls run in parallel were negative.

Solution phase PCR was used to optimise all N, F and H gene primer sets. No reason is given for why no standard curve was prepared for the N-gene.

The reader's assumption is that, following this assay optimisation, patients and controls were also analysed using F and H genes, with possible amplification of the N-gene. However, the paper does not explicitly states this, nor does it state what has happened to the analysis of the N-gene. No mention is made of the technical controls. Essentially, no data are being presented:

Seventy of 91 affected children were positive for MV compared with four of 70 controls as analysed by TaqMan RT-PCR (table 2). MV copy number in positive biopsies was generally low, but ranged from 1 to 3×10^5 copies of MV/ng of total RNA. Of the paediatric control group, MV was not detected in normal children or children with isolated ileal LNH. However, four of 26 appendicectomy samples harboured the MV genome (table 2).

1-3x10^5 copies of RNA/ng total RNA is not low. It is the equivalent of a high copy number cellular gene such as GAPDH. This level of expression is easily detectable by RT-qPCR, and certainly is way above a level that could be described, as Dr Hepner does, as "*hovering around its detection threshold*".

4. Consistency and reproducibility

Dr Hepner quotes from an international collaborative study that reports laboratories can differ in sensitivity by as much as 1,000-fold in terms of the ability to detect MV sequences in clinical samples [8]. She fails to quote a second

finding, which asserts that PCR findings, positive or negative, are questionable if they are not supported by data demonstrating the overall sensitivity of the assay. The O'Leary paper singularly fails to do this, as no amplification efficiency or sensitivity data are presented, and no reference is made to whether the F-gene, the H-gene, or both are being detected, whether results were concordant and how the MV copy number was calculated. There is no information with regards to threshold cycles, number of replicates or the number of repeat assays. The absence of such data makes the results pointless and does not allow their meaningful interpretation. In this context it is interesting to note Dr Hepner's comment that "*For MV studies, therefore, it is advisable to establish detection limits for a particular assay design as well as particular clinical materia*l", since she does not apply this criterion to the O'Leary paper.

Dr Hepner's asserts that "*one must not discount data demonstrating presence of MV in clinical materials due to problems of repeatability … as long as the original study … performed a properly controlled and technically accurate study*". There can be no doubt that the O'Leary study does not fulfil either of these two criteria.

5. Are the criticisms of the Uhlmann study reasonable?

Dr Hepner's criticism of the Afzal study [9] is partly acceptable, but too sweeping in its conclusion. That study justifies its use of leukocytes by referring to a report of the presence of MV in one of two blood samples of autistic regression cases [10]. The authors also had sight of unpublished assay results from O'Leary and Bradstreet, in which they apparently detected MV at high frequency in blood samples. Therefore, the authors' choice of blood to screen for the presence of MV seems to be a reasonable one. The study aims to reproduce O'Leary's experimental conditions as accurately as possible, hence provides a useful baseline for comparison of results. In addition, Afzal does not "*conclude that the Uhlmann study is not credible*". The conclusion of Afzal's paper is that they could not verify the finding of previous studies (including Uhlmann's) that predicted persistence of measles virus in autistic children with developmental regression.

The purpose of the D'Souza publication [3] was to subject the real-time protocol and reagents used by Prof O'Leary's laboratory to a rigorous comparison and determine the quality of that assay. The data presented show unambiguously that the O'Leary assay is significantly flawed and is unable to detect reliably the presence of MV RNA.

Surprisingly, Dr Hepner states that "*melting curves in the Uhlmann study were consistent with true MV amplification*". This is peculiar for two reasons: (1) melting curves are not mentioned in that paper because (2) that study involved TaqMan chemistry, whereas melt curves are used with SYBR Green chemistry. Since the hybridised TaqMan probe is degraded during polymerisation, there is no labelled amplicon that could be subjected to melt curve analysis. This comment reveals a fundamental ignorance of basic real-time PCR technology.

Dr Hepner's asserts that "*One may conclude … that these primers are not appropriate primers for PBMC derived RNA, or that PBMCs do not have detectable levels of MV. One may not draw conclusions about the credibility of the Uhlmann study due to lack of comparable testing*". While testing PBMC's may not be the same as testing biopsy samples, the point both D'Souza and Afzal make is that given the reported levels of MV in the Uhlmann studies, one would expect to see MV everywhere, even in PBMCs. The fact that MV was not detected in PBMCs further undermines Uhlmann's theory. Following the extraction and quality assessment (testing for inhibitors and integrity) of RNA, primer performance is not tissue-dependent; hence to conclude that a set of primers is not appropriate for detecting a RNA target from any particular tissue is wrong. The observation by D'Souza of amplification of non-specific targets, on the other hand, does provide clear evidence for a lack of specificity of the O'Leary assay and strongly suggests that this lack of specificity is a characteristic of the primers, rather than of the source of the RNA.

6. Supportive data for MV RNA presence

Dr Hepner describes data from a poster abstract presented at the International Meeting For Autism Research (2006), in which "*70/82 ileal biopsies from developmentally delayed children with GI symptoms were positive by RT -nested PCR for MV F gene. Vaccine strain specificity was conferred in a percentage of these samples using nucleotide sequencing. Multiple primer sets were used in this study in an attempt to optimise conditions for a PCR based assay that can be used uniformly and by other investigators who extract target template from GI biopsy tissue*".

Dr Hepner is a co-author on this abstract, hence is not citing independent supportive data.

The abstract does not provide any information concerning the RT-PCR assay, its sensitivity, specificity or efficiency. Strangely, there appear to be no biological controls (healthy volunteers) included in the study. A review of the peer-reviewed literature does not show any publication arising from this poster. However, I have not seen the poster itself.

A more exhaustive literature search for the authors does not reveal any high level of expertise, either in the field of autism or in RT-PCR: A. Krigsman: 1 publication (2002) on laryngeal dysfunction; K(D) Hepner: 2 publications (2000, 2002) on scaffold proteins. Only one paper (2002) mentions PCR, and then as a method for amplifying DNA, not RT-PCR for quantitating mRNA levels. J Segal and SJ Walker have published one paper in this general area [11], but this did not use RT-PCR. SJ Walker has published one short review in this general area [12].

7. References

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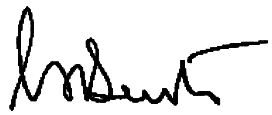

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