Testimony before Congressional Oversight Committee on Autism and Immunisation by John J. O'Leary MD, PhD, MSc, MRCPath.

Mr. Chairman and members of the committee,

The purpose of this testimony is to report the scientific results in a series of children with autistic enterocolitis. Nothing in this testimony should be construed as anti-vaccine, rather it encourages safe vaccination strategies. The opinions expressed in this text and attendant presentation represent those of the author, and are not representative of any organisation or institution.

The studies were undertaken following an approach made to me by Dr. Andrew Wakefield (who has submitted independent testimony). The studies represent a trans-national, multi-disciplinary research programme aimed at elucidating the causes and pathogenesis of inflammatory bowel diseases and developmental disorders of childhood.

Biopsy material for this study was provided by Dr. Wakefield and presented to my laboratory using "blinded protocols". Unique accession numbers were then assigned to each case to maintain patient and diagnostic anonymity. Senior scientists and technicians have carried out the research work at a dedicated state of the art molecular biology facility.

Dr. Wakefield posed three questions to our group in relation to autistic enterocolitis:

- Was measles virus present in gut biopsies of affected children?
- Where was measles virus located in gut biopsies of affected children?
- How much virus was present in gut biopsies of affected children?

Following scientific discussions it was decided to augment this panel of questions by including two additional important questions:

- Could measles virus genomes be sequenced from gut biopsies from children with autistic enterocolitis?
- Could different molecular technologies be employed to confirm the detection of measles virus genomes in affected children?

Before commencement of the project a standard operating procedure (SOP) was written in relation to handling of samples, extraction of nucleic acid and performance of molecular virology screening assays. The assays used in this study were:

- In-situ hybridisation (with and without tyramide signal amplification [TSA]).
- In-cell PCR
- Solution phase PCR
- TaqMan quantitative PCR
- DNA sequencing

Specific regions of the measles RNA genome were selected as detection targets. These included the haemagglutinin (H), nucleocapsid (N) and fusion (F) regions of the measles genome.

Strict anti-contamination procedures were adopted throughout the study to prevent false positive results being generated. These included separate and isolated facilities for nucleic acid extraction, PCR amplification, in-situ hybridisation and DNA sequencing.

TECHNOLOGIES:

In-situ hybridisation:

This technique allows localisation and visualisation of genetic sequences (DNA and RNA) in cells and tissue sections. The in-situ hybridisation assays employed cloned cDNA (copy DNA) fragments of the H, N and F region of the measles genome. Cloned fragments were labelled with biotin and /or digoxigenin using standard nick translation technology. Hybridisation (specific attachment) of probe to the target sequence in cells and tissues was carried out using conventional chemistries. Detection of the formed hybrid was achieved using standard immunocytochemical techniques. In all cases tyramide signal amplification (TSA) was applied to increase detection sensitivity.

One-step immunocytochemical detection has a sensitivity of 50 genome copies per cell; three step immunocytochemical detection has a sensitivity of 10 – 15 copies per cell: However, TSA achieves single copy viral gene detection in cells and tissue sections.

In-situ hybridisation assays were performed on serial sections of gut biopsies from affected children. Hybridisation efficiency was assessed using a conserved human repeat sequence (i.e. present in every cell). Negative control probes included Human Papilloma virus types 16 and 18 and Human Herpes Virus 8.

Immunocytochemical and detection controls were included in all assays.

Optimisation experiments were carried out using measles virus infected Vero cells and brain biopsy material from patients affected with sub-acute sclerosing pan-encephalitis (SSPE), which is caused by measles virus.

In-cell PCR:

This technique allows the investigator to amplify (make copies of) DNA and RNA in cells and tissue sections with a detection sensitivity of one viral or mammalian genome copy per cell. The location of the virus within the tissue can be identified. In addition, problems with DNA and RNA contamination are not encountered using this method, because only DNA/RNA present in tissue sections (i.e. either inside or outside cells) will be amplified and localised. (Figure 1).

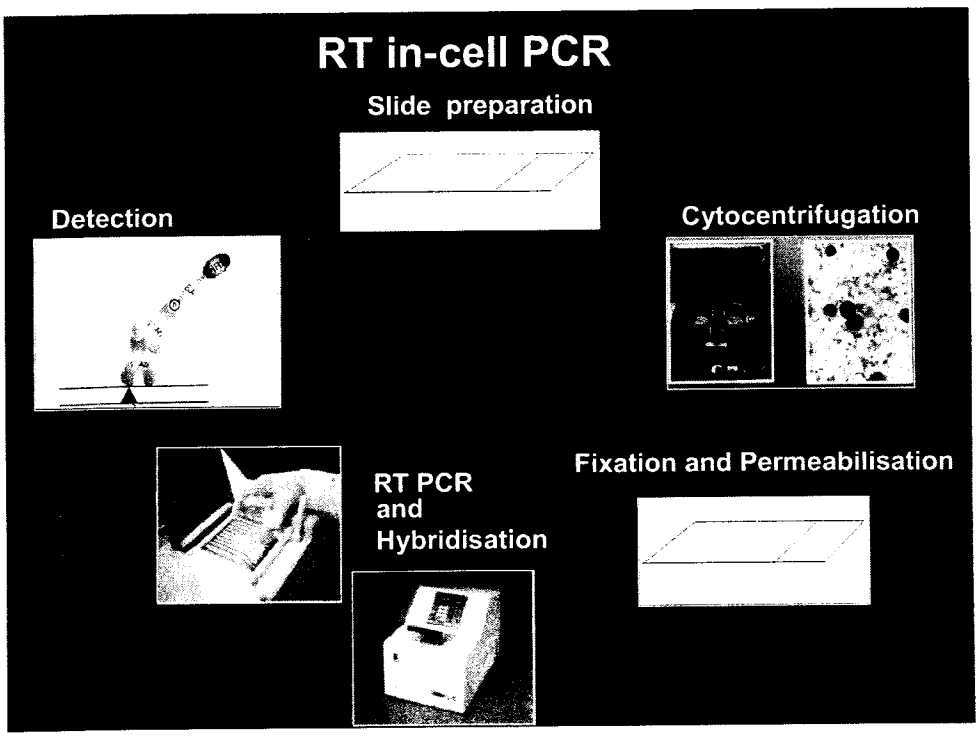


Figure 1: Schematic representation of RT in-cell PCR

In these cases, measles virus RNA was amplified using in-cell RT-PCR (reverse transcriptase PCR). This technology employs a polymerase chain reaction (PCR) step (in order to make copies of the RNA molecule) and a hybridisation step using a labelled probe to detect the newly formed amplicon (gene copies).

Optimisation experiments were carried out using measles virus infected Vero cells and measles-infected brain biopsy material from patients with sub-acute sclerosing pan-encephalitis (SSPE).

Four to six serial sections of gut biopsies from affected children were examined for the presence of measles virus, while including appropriate controls, i.e.

- omission of reverse transcriptase enzyme,
- omission of DNA polymerase,

- using irrelevant primers
- immunocytochemical and detection controls.

Solution phase PCR:

For affected children from whom frozen biopsy material was available, solution phase PCR using primers to H, N, and F regions of the measles virus genome was performed. (Figure 2).

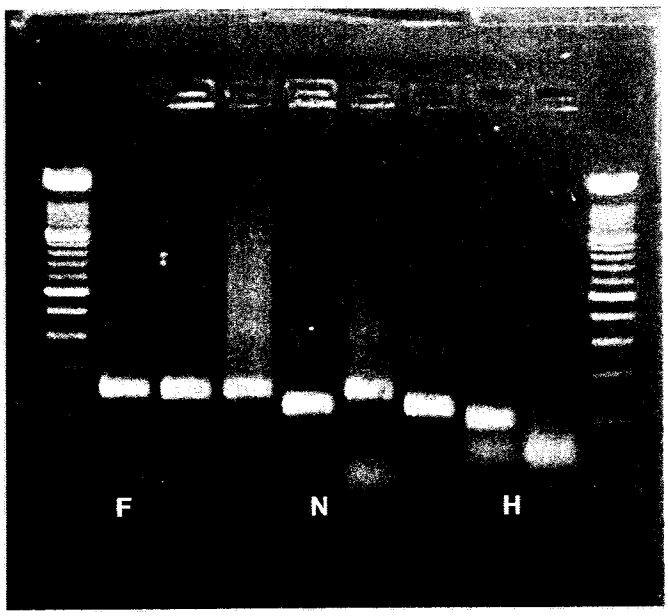


Figure 2 Solution phase PCR of F,N and H genes of measles virus.

Optimisation experiments were carried out using measles virus infected Vero cells and brain biopsy material from patients affected with sub-acute sclerosing pan-encephalitis (SSPE), which is caused by measles virus.

The detection sensitivity of single round PCR is 15 viral copy RNA equivalents (cRNA) in 10^5 RNA sequences.

Taq Man quantitative PCR:

This technique allows automated quantitative PCR analysis of RNA and DNA gene sequences. My laboratory has been involved with this technology for approximately 5–6 years. TaqMan PCR utilises two primers (as in conventional

solution phase PCR), but in addition uses a probe labelled at one end with a fluorescent reporter molecule and at the other end by a fluorescent quencher molecule. These molecules are chosen so that the reporter emits fluorescence at the specific wavelength that the quencher will absorb fluorescent light. No fluorescence can be detected when the probe is intact due to the proximity of the reporter molecule to the quencher. The probe is designed so it will bind to the target sequence between the two primers.
It is also important to note that TaqMan PCR utilises a characteristic of the enzyme Taq polymerase, namely its 5' nuclease activity.

If the target sequence of interest is present, then both primers anneal (stick) to the ends of the target sequence. At the same time hybridisation (sticking) of the TaqMan probe occurs. During the extension phase of PCR, the TaqMan probe is displaced and cleaved (broken) with release of the reporter molecule into solution away from the quencher sequence. For each new copy of the gene that is made, one reporter molecule is released which can be monitored in a specifically designed sequence detector (7700 sequence detector (PE Biosystems)).

The assay is entirely sequence specific and does not yield false positive results. In addition, it is a closed tube assay, which minimises potential contamination events. (Figure 3).

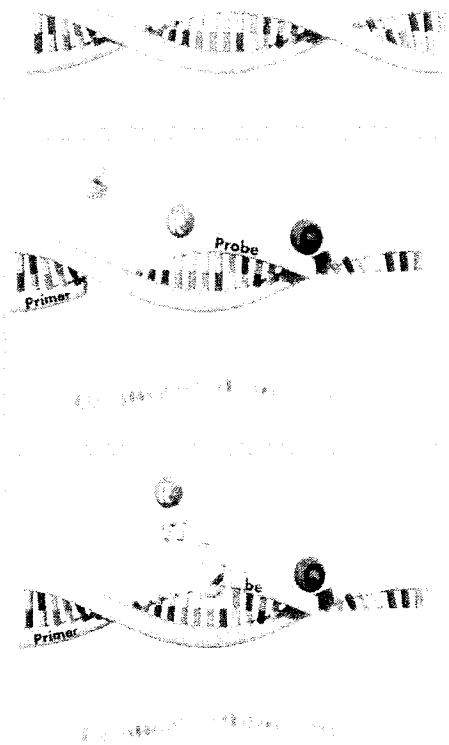
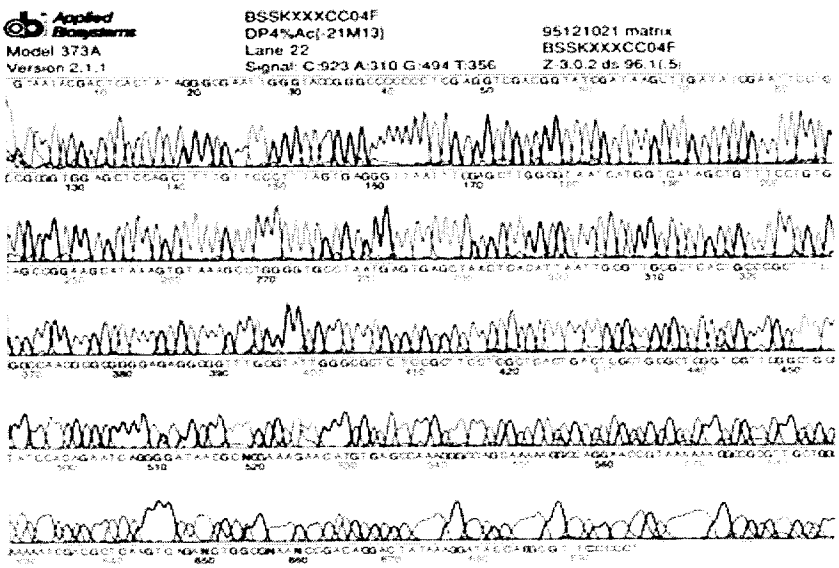


Figure 3 Schematic representation of TaqMan PCR

DNA sequencing:

Sequence confirmation of measles virus genomes was carried out using the ABI Prism 310 capillary sequencer (PE Biosystems). BigDye terminator chemistry and cycle sequencing was employed to validate derived sequences. Positive and negative strand sequencing was performed with subsequent alignment of genetic sequences using BLAST search software. (Figure 4).



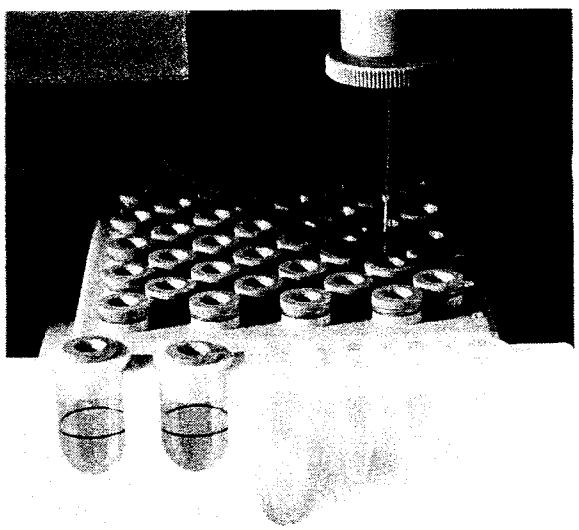
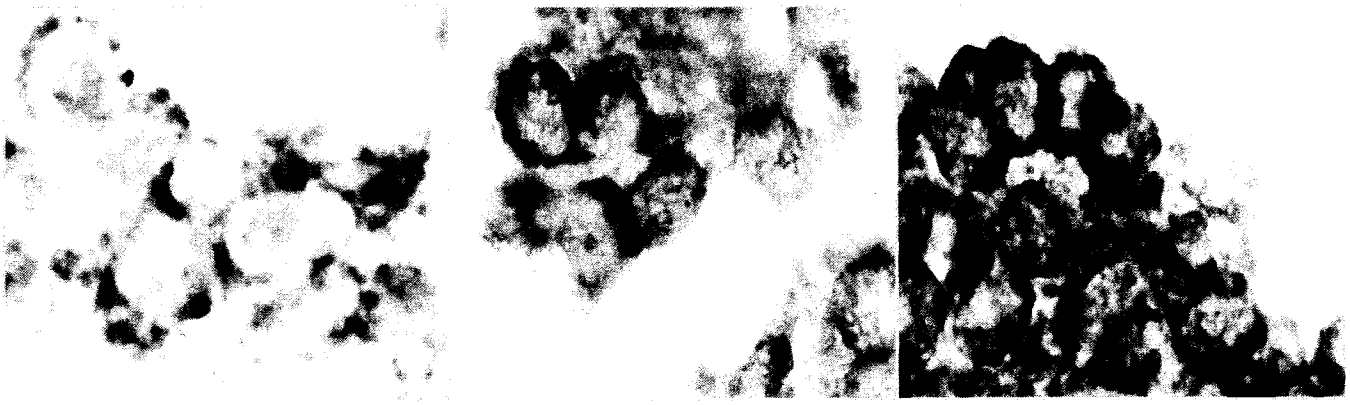


Figure 4. Electropherogram readout of a sequencing reaction. Bottom plate illustrates capillary 310 platform.

RESULTS:

I. Optimisation experiments



1μg/ml **1.5μg/ml** **2μg/ml**

Figure 5 **(above) demonstrates the optimisation experiments for RT-in-cell-PCR assays with measles virus transfected Vero cells. Copies of measles virus RNA can be seen in the cytoplasm of the cells. The intensity of the signal is dependent on the concentration of probe used for the hybridisation component of the assay.**

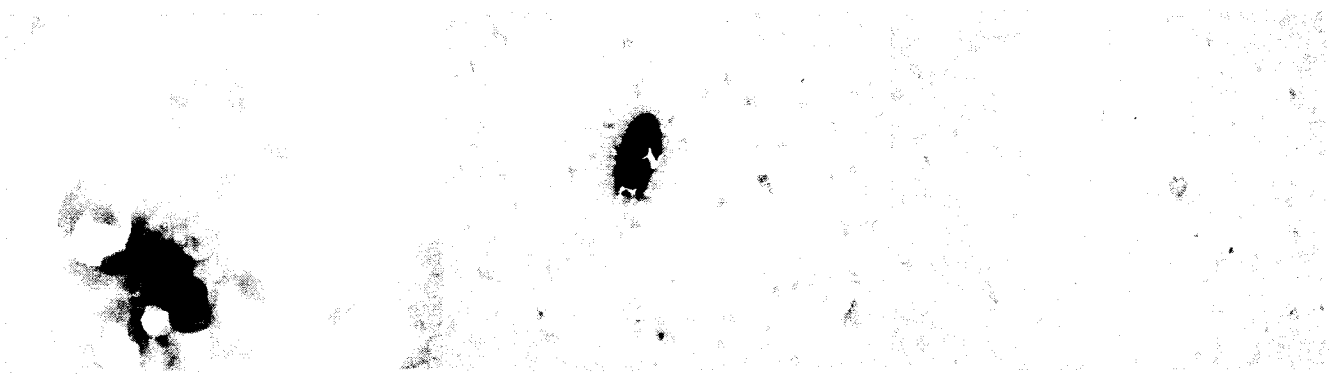


Figure 6 **(above) demonstrates in-cell PCR results in patients with SSPE. Note the presence of measles virus genomes in brain tissue of affected patients.**

II Biopsy results

Using RT in-cell PCR and in-situ hybridisation with TSA, we are able to identify measles virus genomes in follicle centres of lymphoid aggregates of gut biopsies from children with autistic enterocolitis. The signals obtained (see Figure 7) appear to be extra cellular and fibrillary in nature. Localisation of measles virus genomes was confirmed on serial sections from the same patient. In each biopsy, 1–3 loci of amplification were identified; indicating low measles virus copy number. The results must be taken in the context of formalin fixation, which, by its nature degrades RNA molecules in cells and tissue sections.

The fibrillary pattern seen by in-cell PCR is similar to immunocytochemical results obtained for measles virus N-protein immunocytochemistry (see Figure 7).

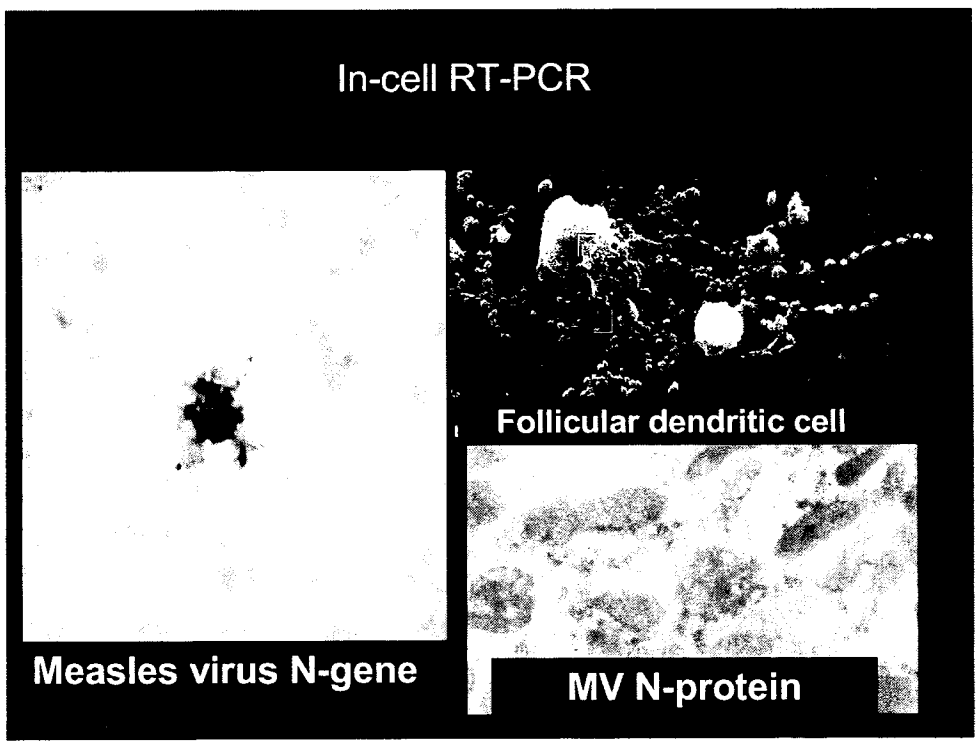


Figure 7 **Left panel illustrating RT in-cell PCR results. Note the fibrillary quality of the signal, which is extracellular.**
Top right panel: published electron microscopic image of a dendritic cell showing fibrillary processes of the dendritic cell matrix.
Bottom right panel demonstrating measles virus N-protein in a follicle centre. Brown/orange stain indicates positivity.

Measles virus genomes were identifiable by standard solution phase PCR (see Figure 8) using fresh frozen gut biopsies from children with autistic enterocolitis.

1: Patient 2:MV-F-protein (150bp)
2: Patient 3:MV-F-protein (150bp)
3: Patient 2 :MV-H-protein (150bp)
4: Patient 3: MV-H-protein (150bp)
5: pos.control:MV-H-protein
6: pos.control:MV-F-protein
7: pos.PCR control (PDH Junc)
8: neg.control

Figure 8 Measles virus – solution phase PCR of patient samples.

Using TaqMan PCR, we have been able to quantify the measles virus copy number per 1,000 mucosal cells using 'gene dosage correction formulations'. The copy number of measles virus in gut biopsies from children with autistic enterocolitis is low, at approximately 30 – 50 measles virus genomes per 2,000 mucosal cells (including gut epithelial, lymphoid and dendritic cells) (Figure 9).

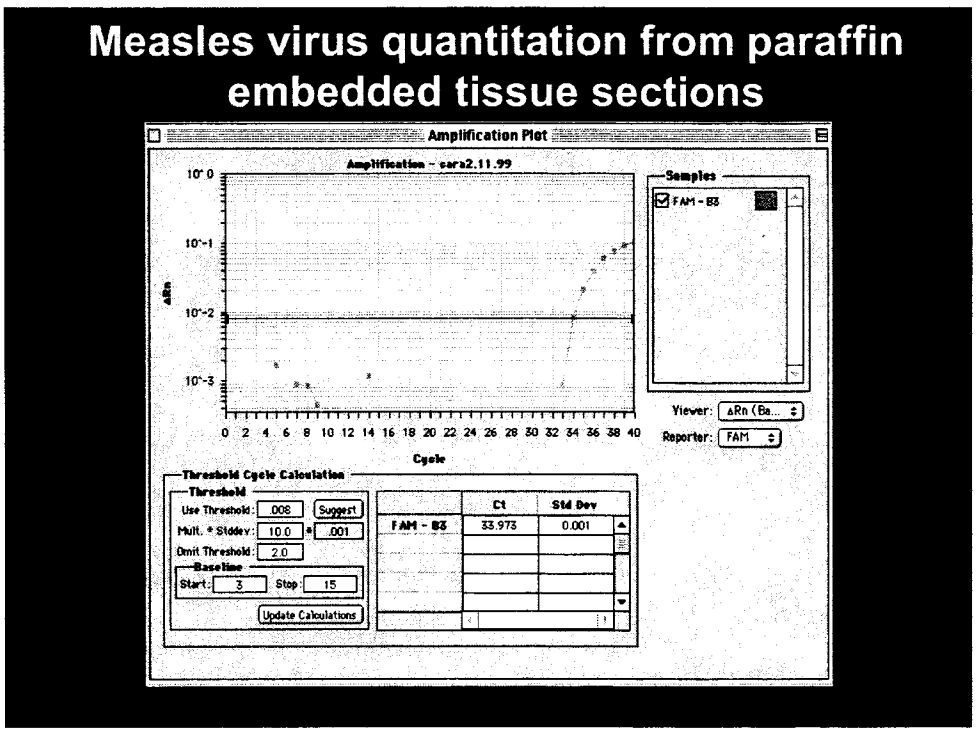


Figure 9 TaqMan PCR amplification plot of a measles virus amplicon from a paraffin embedded tissue section of an affected child.

Confirmation of the presence of measles virus genomes was achieved using positive and negative strand sequencing of cDNA measles amplicons (see Figure 10).

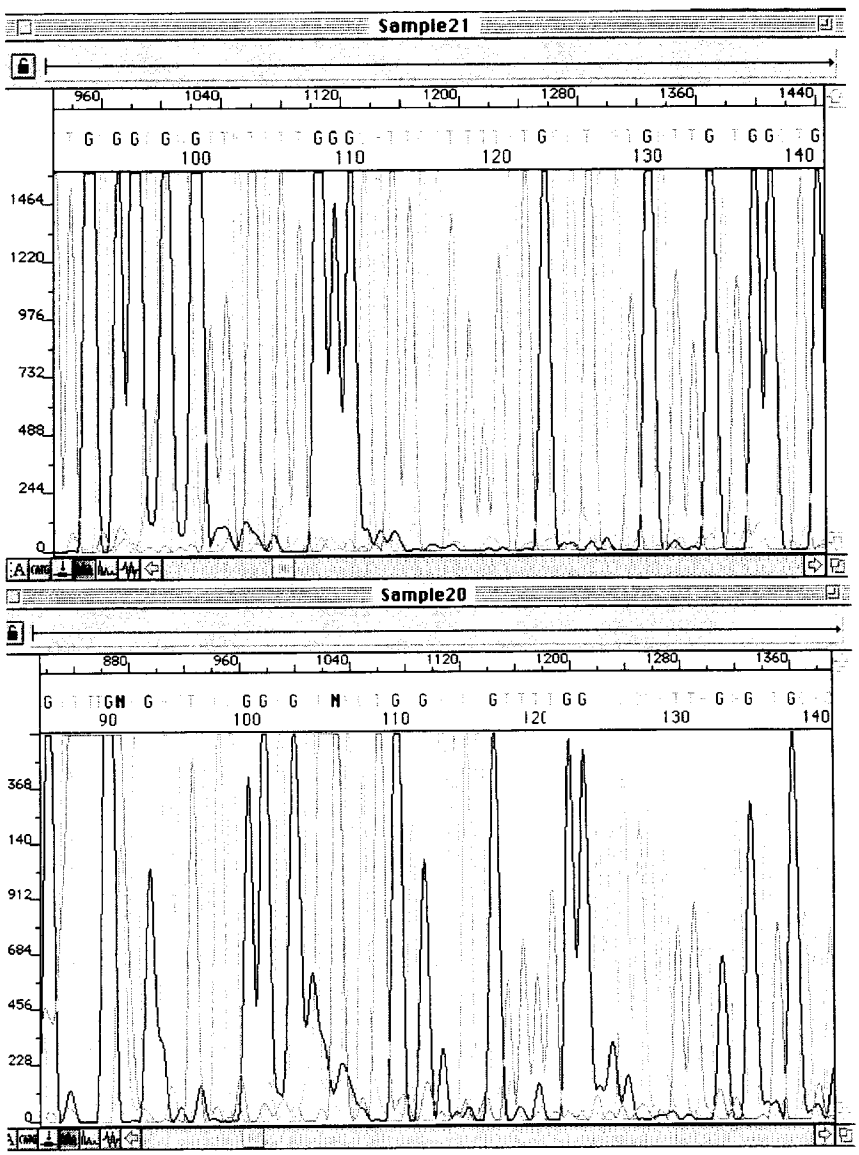


Figure 10 **Positive and negative strand sequencing of measles virus F region in an affected child.**

Following investigation by scientists and technicians in our department, the subsequent results have been obtained:

Autistic enterocolitis children
Twenty four of 25 (96%) autistic children were positive for measles virus including 2 children from the US who were included in this analysis.

Control children
One of 15 children (6.6%) was positive for measles virus.

CONCLUSION:

Using different molecular biological technologies we have been able to identify, localise, quantitate, and sequence measles virus genomes in gut biopsies of children with autistic enterocolitis.

The question that now remains to be answered is:-
What is measles virus doing in lymphoid germinal centres of children with autistic enterocolitis?