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Measles Vaccination and Antibody Response in Autism Spectrum Disorders

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Abstract

Objective: To test the hypothesis that measles vaccination was involved in the pathogenesis of ASD as evidenced by signs of a persistent measles infection or abnormally persistent immune response shown by circulating measles virus or raised antibody titres in MMR vaccinated children with ASD compared with controls .

Design: Case-control study community based

Methods: A community sample of vaccinated children aged 10-12 years in the UK with ASD (N=98) and two control groups of similar age, one with special educational needs but no ASD (N=52) and one typically developing group (N=90), were tested for measles virus and antibody response to measles in serum.

Results: No difference was found between cases and controls for measles antibody response. There was no dose response relationship between autism symptoms and antibody levels. Measles virus nucleic acid was amplified by RT-PCR in PMBC from one case with autism and two typically developing children. There was no evidence of a differential response to measles virus or the measles component of the MMR in children with ASD, with or without regression, and controls who had either one or two doses of MMR. Only one child from the control group had clinical symptoms of a possible enterocolitis.

KEYWORDS: Autism, Autism spectrum disorders, MMR Vaccination, SNAP

Word Count 2953

Introduction

Recent studies of the prevalence of autism spectrum disorders (ASD) have found rates between 6 and 12 per thousand, significantly higher than previous estimates, depending on the strictness with which the diagnostic criteria are applied[1,2,3]. Although widening of diagnostic concept, improved ascertainment and other methodological aspects of more recent studies are likely to be major causes of the increased rate, and despite the fact that autism is known to have a strong genetic basis; concerns about environmental risk factors for an increased prevalence have inevitably been raised.

In 1998, a report of a small case series of 12 children and no control group suggested that measles, mumps and rubella (MMR) vaccination might be linked to the development of autism spectrum disorders [4.] A subsequent larger case series described a condition referred to as 'autism enterocolitis', which was postulated to be associated with MMR vaccination and specifically with regression in autism [5].

Several epidemiological studies found no association between MMR vaccination and autism spectrum disorder [6,7,8]. , however, fear about MMR vaccination resulted in reduction of uptake of combined MMR vaccine, which fell from 92% in 1995-96 to 80% by 2004[9], risking exposure of the population to a measles epidemic and outbreaks in susceptible groups [10]. There continues to be an impact on parents of children with autism [11] and general public concern of risk which is reflected in parental decisions about MMR vaccination [12],[13]

Elevated levels of measles antibodies have been reported in autism[14]. Two laboratories have reported the detection of measles virus, one by conventional reverse transcriptase polymerase chain reaction (RT-PCR) in 3 cases of autism[15] and another by real-time Taqman PCR[16], the latter in intestinal samples of

75/91 patients with ASD compared to 5/70 control patients. The origin and characterisation of the fragments of measles virus genome described in these studies has not been established and concerns about the scientific methods employed widely expressed. Two recent studies have failed to find measles virus genome by real-time PCR in children with ASD compared with controls in peripheral blood mononuclear cells (PBMC) rather than gut mucosal samples. [17,18]

We took advantage of a new geographically defined study of the prevalence of ASD (Special Needs and Autism Project; SNAP)[1], to test the hypothesis that measles vaccine was involved in the pathogenesis of ASD as evidenced by signs of a persistent measles infection or abnormally persistent immune response shown by circulating measles virus or raised antibody titres in MMR vaccinated children with ASD compared with controls in particular in children with ASD and a history of regression. Measles virus replicates in a range of cells during infection including the upper respiratory tract, intestinal cells, several T cell lineages and macrophages. Replication occurs for similar periods in these different sites. An earlier study had suggested virus detectable in peripheral blood mononuclear cells (PBMC) PCR in blood samples in children with ASD [15] . We used PBMC in this study as proxy for gut mucosal cells which were not obtained for ethical reasons.

Methods

Participants. The population studied is a cohort of 56,946 children born between July 1st 1990 and December 31st 1991 from 12 districts in the South Thames region of the UK. At age 9-10 years children with a statement of special educational needs (SEN) (1733; 218 of whom had a local ASD diagnosis) or a local diagnosis of ASD but no SEN statement (37) were screened using the Social Communication Questionnaire (SCQ)[19]. Stratification by local diagnosis and high, medium and low SCQ score was used to derive a subset (255) who received an in-depth diagnostic

assessment (see figure 1 for a flow chart of the process and Baird et al for further explanation). The diagnostic assessment included standardized clinical observation (Autism Diagnostic Observation Schedule – Generic (ADOS-G))[20] and parent interview assessments of autism symptoms (Autism Diagnostic Interview-Revised (ADI-R))[21], language and IQ, psychiatric comorbidity and a medical examination (see Table 1). Children were classified using ICD-10 research criteria as childhood autism, other ASD or no ASD by clinical consensus using all sources of information. The ASD group was divided into a 'broad ASD' and 'narrow autism' group, the latter defined as meeting autism criteria on the ADI-R, the ADOS-G and clinical consensus of ICD-10 childhood autism and the broad ASD defined as all other cases meeting clinical consensus of any ASD. The total number of ICD-10 autism symptoms was recorded. Those who experienced 'regression' were divided into a 'definite language regression group' defined as the loss of 5 or more words used communicatively during a 3 month period and a 'lower level regression' group who had not achieved the 5 word stage at the time of regression but had reported regression of words or skills in social communicative or play behaviour. The 'no ASD' group had a variety of diagnoses, learning difficulties, specific language or literacy impairments, ADHD, cerebral palsy, deafness and visual impairment.

After obtaining consent, of the 255 children seen for an in-depth assessment, sufficient blood suitable for analysis was collected from 101 cases with an ASD diagnosis (mean age = 11.6 years, SD .88), and 54 SEN controls with a non-ASD diagnosis (mean age = 12.7 years, SD .88). The age span reflects the time scale of the diagnostic project.

A further 98 typically developing (TD) controls, born at the same time, attending two mainstream schools within the same geographic area who did not have a statement of special educational needs and who consented to venepuncture were

recruited. The SCQ was used to screen out possible cases of autism and eight cases were subsequently excluded from analysis on the basis of scores of at least 15, the cut-off recommended for identifying likely cases of ASD[19]. The mean age of the 92 TD controls was 12.2 years (SD .33).

Gastrointestinal symptoms (GIS) reflecting the presentation of GI symptom constellations in general clinical paediatric practice were assessed using a 22 item questionnaire completed by the main caregiver (in preparation) Current (in the last three months) and past symptoms were elicited. A 'possible enterocolitis' group was constructed from the presence of 2 or more of the following 5 current gastro intestinal symptoms : current persistent diarrhoea (defined as loose/watery stools three or more times a day >14 days), current persistent vomiting (occurring at least once per day or more than five times in a week), current weight loss, current persistent abdominal pain (3 or more episodes severe enough to interfere with activity); current blood in stool; plus past *diarrhoea >14 days duration* and excluding current constipation.

Vaccination.(see figure 1) Information about MMR vaccination was obtained about all children using district records, parent records, and GPs. 235 children had received the first MMR vaccination, 98 (97% of the group) with ASD, 52 (96%) SEN controls, and 85 (94%) TD controls. 106 children received the stage 2 MMR vaccination (first introduced in 1996) (35 (36%) children with ASD, 18 (35%) SEN controls and 53 (62%) TD controls). Five children who lacked evidence of at least one MMR vaccination were excluded from the analysis.

Studies show 95% seroconversion for measles after the first MMR vaccination with the second dose of MMR converting most of those who did not with the first vaccination and inducing only a transient rise in antibody proportional to the earlier response in earlier responders[22]. Thus it is justifiable to include every child who had

had at least one MMR vaccination in a case-control comparison of vaccinated children: 98 ASD cases (32 narrow autism; 66 broad ASD); 52 SEN controls and 90 mainstream (TD) controls. However, for completeness, children who had had only one MMR and those who had had two MMR vaccinations were analysed separately and then were combined . For some analyses, the SEN non-ASD controls and TD controls are compared separately and then in combination to form a total control group of 142. The 98 ASD cases are analysed as broad ASD and narrow autism separately and in combination.

Laboratory Tests. Clotted and anti-coagulated blood samples (in ethylenediaminetetraacetic acid) were couriered to the laboratory on the day of collection for processing. Serum was separated and stored at -20°C until tested for antibody. Samples were processed using the amplicor kit and then stored at -70 °C until tested for measles virus. Samples were batched and the laboratory was blind to case control status.

Genome Detection. Detection of measles virus in the EDTA sample was conducted on peripheral mononuclear cells after concentration using the amplicor whole blood preparation kit (Roche). Satisfactory EDTA samples for this were available from 94/98 ASD cases and 130/142 SEN and TD controls. Samples were tested for presence of measles genome after extraction of RNA using the Magnapure extractor. Three RT-PCR assays were used; published assays for M gene[23] and N gene RT-PCRs were used [24]. A RT-PCR for H gene using AB1 PRISM 7000 sequence detector platform (TaqMan) was developed [25]. Assays were run for 40 cycles and data analysed according to the manufacturer's instructions. For the real-time assay, the following primers and probes were used:

Gene	**Primer**	**Position**	**Sequence (5'-3')**	**Product**
H gene	Forward	117-140	GGCTGTTCTGTTTGTCATGTTTGT	68

Reverse	161-184	GATGAAGTCTAATGCCTGCAATGG
Probe	141-156	CAACCCGATCAAGCTC

The sensitivity of the assay was determined to be 2 genome copies. Samples were tested to ensure they were adequate using a β_2 microglobulin housekeeping gene PCR with a sensitivity of 10 genome copies per reaction mixture.

Antibody Studies. Serum samples were tested for measles IgG antibody by plaque reduction neutralisation test (PRN). PRN was chosen because recent evaluation has demonstrated its greater sensitivity over commercially available EIA tests[26]. Measles antibody was quantified in international units to control variation using the international reference standard sera for PRN[27].

Statistics. All summary statistics and analyses of antibody response are based on $\log_{10}$ transformed milliInternational Units per milliLitre (mIU/mL) and undertaken in Stata 9 [28]. Having tested for homogeneity of variance (Bartlett test), we report ANOVA F and Scheffé tests for the 4-group comparison of Typically Developing (TD), SEN-No ASD, Broad ASD and Autism. In addition, in view of the variety of specific alternative hypotheses proposed, we report both further pairwise comparisons of combined groups, a group defined by regressive autism and a linear trend test over the quintiles of the ICD-10 autism symptom score. Results are also reported for these additional analyses using Wilcoxon rank based tests[29] that enable the inclusion of subjects with no detectable antibody response (coded as 0). P-values from these rank based tests are denoted p*. All p-values and confidence intervals for these additional paired comparisons are nominal with no correction for multiple testing.

Results

Descriptive statistics of TD, SEN, broad ASD and narrow autism groups are shown in Table 1.

Measles Virus Assays. Samples from all cases contained detectable β_2 microglobulin gene by PCR. All samples were negative in measles H gene RT-PCR assay. 56 samples (based on availability of sufficient nucleic acid) were also tested and negative in the N gene RT-PCR. One sample from a case and 2 samples from controls were reactive in the M gene PCR. These PCR products were sequenced; a genotype C2 measles strain was characterised in one case (who had narrow autism but no regression history) and a measles vaccine strain and a D6 strain from 2 typically developing mainstream controls. These sequences were unlike any previous isolates seen in the laboratory. The results were not repeatable; the 3 reactive samples were negative when retested in the M gene PCR.

Antibody Response to Measles. Eight subjects (1 typically developing, 5 SEN Non ASD, 1 broad ASD and 1 narrow autism) who had received MMR vaccination had no detectable measles IgG antibody by PRN suggesting that the attenuated measles virus did not replicate and triggered no immunological response. There was no difference in mean $\log_{10}$ (mIU/mL) measles titre between those with one or two MMR vaccinations (difference = 0.00, CI(-.12,.11), p=.94, p*=.62).

Figure 2 shows the similarity of distributions of measles PRN responses by group, combined by MMR number. The plots give no indication of extreme titres in the ASD and autism groups that fall outside of the distribution among the controls. The overall difference of means test indicated no significant differences (F(3,223) p=.13) with the most significant of the 6 Scheffé paired comparisons giving p=.23. The corresponding tests for those with a single MMR were F(3,126) p=.20 with most significant Scheffé p=.20, and for those with two MMR were F(3,93) p=.66 with most significant Scheffé p=.74.

The combined control group mean $\log_{10}$-titre was not significantly lower than the narrow autism group (difference 0.05 CI(-0.08,0.18), (F(1,194) p=0.45, p*=0.26), nor the ASD group (difference 0.08 CI(-0.07,0.25), F(1,160), p=0.29, p*=0.26) nor the combined autism/ASD group (difference 0.06, (-0.05,0.17), F(1,225), p=0.27, p*=0.26). This comparison of the combined case and control groups had 80% power to detect a mean titre difference of 45% (or 0.16 $\log_{10}$(mIU/mL). Within the autism groups there was no trend of PRN response over ICD-10 symptom quintiles (p=0.99; p*=0.63).

Regression was reported in 23 children with ASD but PRN titres were not significantly higher among these than combined controls (difference –0.12 CI (-0.30,0.06), F(1,162) p=0.18, p*=0.33).

'Possible 'enterocolitis' as defined above, was found in only I child who did not have ASD or regression.. He had current and past diarrhoea and abdominal pain and was in the combined control group. No child had a previous diagnosis of inflammatory bowel disorder.

Discussion

No difference was detected in the measles antibody distribution or in measles virus in ASD cases and controls whether the children had received the first, second or both MMR vaccinations. This remained true when the analysis was restricted to ASD cases with a history of regression. Only one child had symptoms of a possible enterocolitis and this was in the control group.

This is one of three virological case control studies which have failed to demonstrate any association between measles vaccination and ASD using well validated techniques[17,18]. In the D'Souza study[18], children were 26-30 months from vaccination contrasting with approximately 9 years in this study with identical conclusions. The report from D'Souza et al. also describes an exhaustive validation

of the molecular detection methods used in the only study to detect measles genome in ASD cases[16] demonstrating that the methods used can generate false positive results.

The strengths of this study are that the cases with ASD were from a well characterised community, not clinic, derived sample. The sample is the largest reported.. Regression was clearly defined. The diagnostic process allowed a 'dose response' of ICD10 symptoms to antibody titre to be analysed. All children had well documented vaccination history. A highly sensitive methodology was used for measles antibody assay. The laboratory techniques employed to collect, extract, store and test samples for measles genome used well-established, block-based RT-PCR assays, which have been shown to be highly sensitive in an international comparative study[24]. Laboratory analysis was conducted blind to case control status. A real-time RT-PCR was also used [25]. This platform was used in earlier studies and although of comparable sensitivity to nested conventional PCR, risk of contamination is reduced. PBMC's were used to look for measles by RT-PCR because they are a site of viral replication in acute measles infection and they have been reported to contain measles genome by RT-PCR in a small number of autism children[15].

There are two possible explanations for the finding of one RT-PCR reactive samples in 98 cases of ASD and 2 in the 90 TD children. Immunity to measles is not always complete[30] and measles genome has been detected in the PBMC's of asymptomatic individuals during measles epidemics[31]. C2 and D6 measles genotypes have been detected in the UK population before 2002. The finding could also be due to laboratory cross contamination, which can be problematic with RT-PCR assays.

Limitations of the study:

The TD group were not randomly selected from the total population for reasons of time, convenience and cost. Parents were informed the study was about MMR vaccination and it is possible that a biased group responded to the request to participate. Satisfactory blood samples were not obtained in 100 children, both ASD cases and SEN controls, for a variety of reasons, including refusal by the young person concerned, haemolysation in transport etc. We did not obtain gut mucosal samples for ethical reasons; PBMC were used for measles genome assay justified as a site of known viral replication and appropriate proxy for gut mucosal cells. Gut symptoms were elicited but the children were too old for accurate reporting of retrospective gut symptoms confidently contemporaneous with MMR vaccination. A clinically relevant definition of enterocolitis based on persistent symptoms has therefore been used for this paper.

It is of public health relevance that there is a differential uptake of MMR2 across the groups with both ASD and SEN control groups having lower uptake and hence less exposure to measles virus. This may reflect parental concern about vaccination following a diagnosis of developmental abnormality. Only 29% (20/70) of children who had a local diagnosis of ASD received MMR2 compared with 50% (14/28) who had no local ASD diagnosis.

What is already known on this topic

- Public concern about a putative link between MMR vaccination and ASD has resulted in lower uptake of MMR vaccine
- Epidemiological studies have shown no link between MMR and ASD

What this study adds

- There is no difference between ASD cases and controls in circulating measles genome or measles antibody levels
- There is no evidence of an altered persisting immunological response following either one or two MMR vaccinations in ASD cases with and without a history of regression
- There is no evidence of increased enterocolitis in the ASD group with regression.
- In this cohort, children were less likely to receive MMR2 following diagnosis of a developmental problem.

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Role of Funding Source. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation or writing of the report. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit for publication.

Conflicts of interest

MA and DB have given unpaid advice to lawyers in MMR and MR litigation.
GB has acted as an occasional expert witness for the diagnosis of autism. AP receives royalties from SCQ and ADOS-G instruments. PBS has acted as an expert witness in the matter of MMR/MR vaccine litigation. All other authors have no conflicts of interest.

Ethical Approval

South Thames MREC 00/1/50

Kent & Medway LREC WK153/8/02

Role of authors

GB, ES, TC and DB obtained funding, DB, MA, BT and LJ were responsible for the laboratory tests. TL, SC, and DM collected data and samples. PS was responsible for gastrointestinal assessment. AP had overall responsible for the statistical analysis. All authors contributed to the paper.

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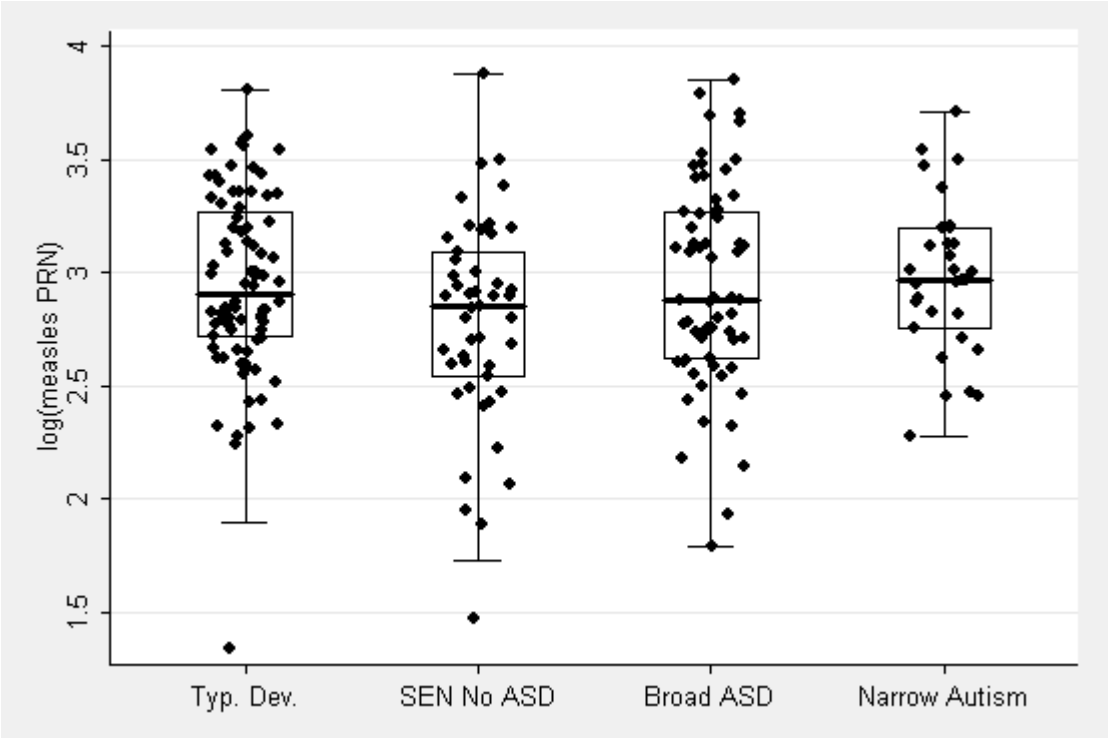
Table 1 Sample Descriptive Statistics: Autism Symptoms and IQ (means (SD))

	TD	SEN (No ASD)	Broad ASD	Narrow Autism
N	90	52	66	32
SCQ score[a]	4.26 (3.59)	9.03 (7.54)	22.03 (6.88)	28.03 (5.06)
ADI-comm[b]	NA	5.37 (3.84)	14.73 (5.57)	18.09 (3.32)
ADI-soc[c]	NA	5.27 (4.85)	19.70 (6.66)	24.69 (3.53)
ADI-rep[d]	NA	1.23 (1.35)	6.00 (3.17)	7.59 (2.17)
ICD-10 sym[e]	NA	1.62 (1.25)	7.21 (2.18)	10.31 (1.64)
ADOS-comm[f]	NA	0.96 (1.10)	2.05 (1.35)	5.59 (2.09)
ADOS-soc[g]	NA	2.84 (2.33)	5.27 (3.10)	10.59 (1.93)
ADOS-rep[h]	NA	0.60 (0.77)	1.74 (1.64)	3.66 (2.12)
IQ	NA	78.46 (20.21)	78.94 (22.49)	63.84 (17.67)
Age in Years	12.2 (0.33)	12.7 (0.89)	11.6 (0.90)	11.7 (0.90)

a SCQ = Social Communication Questionnaire
b ADI-comm = Autism Diagnostic Interview-Revised Communication domain algorithm score (4-5 years)
c ADI-soc = Autism Diagnostic Interview-Revised Reciprocal Social Interaction domain algorithm score (4-5 years)
d ADI-rep = Autism Diagnostic Interview-Revised Repetitive and Stereotyped Behaviours domain algorithm score (4-5 years)
e ICD-10 sym = ICD-10 symptom count (0-12)
f ADOS-comm = Autism Diagnostic Observation Schedule - Generic Communication domain algorithm score
g ADOS-soc = Autism Diagnostic Observation Schedule - Generic Social domain algorithm score
h ADOS-rep = f ADOS-comm = Autism Diagnostic Observation Schedule - Generic Repetitive domain algorithm score

Figure 2 Measles PRN responses for the typical/mainstream, SEN, (broad) ASD and narrow autism groups



$\log_{10}$ (mIU/mL in measles PRN)

Key to boxplot: The box indicates the interquartile range and thick black line the median of each distribution (Geometric means: Typ.Dev. 2.95; SEN No ASD 2.79; Broad ASD 2.94; Narrow Autism 2.98). Whiskers extend to the highest and lowest observed values or, if less extreme, 2.5 times the inter-quartile range from the median.

Figure 1. Sample selection