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Comparative Evaluation of Measles Virus-Specific RT-PCR Methods Through an International Collaborative Study

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Comparison of RT-PCR assays established in house at various places revealed that laboratories could differ in sensitivity by as much as 1,000-fold in terms of the ability to detect measles virus sequences in clinical samples. The study indicates that PCR findings, positive or negative, are questionable if they are not supported by the associated data demonstrating the overall sensitivity of the assay applied. Measles virus-specific RT-PCR-based assays need to be validated using standard virus preparation or nucleic acid-based target templates. A correlation between real-time quantitative PCR and the conventional PCR for measles virus is highly desirable. ***J. Med. Virol. 70:171–176, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: measles virus; RT-PCR; diagnosis

INTRODUCTION

It has been shown that modern methods based on the detection of nucleic acid molecules are extremely powerful tools for demonstrating the presence of microbial agents in diseased tissue. Methods such as polymerase chain reaction (PCR), in situ hybridisation, and in situ PCR are of greater diagnostic sensitivity, specificity, and reproducibility than other methods. Classic techniques such as histopathological examinations of clinical tissues, serological and immunological detection, and transmission electron microscopy provide information on the gross pathological condition of the affected tissue and localisation of the invading microbe in the tissue/cell. When applied at high assay sensitivity, however, they may suffer from misinterpretation or lack of specificity. The use of PCR is attractive because of its robustness, reproducibility, sensitivity, specificity, and the ability to confirm the nature of the nucleic acid detected by determining its sequence.

It has been proposed that measles virus and measles, mumps, and rubella vaccine (MMR) may be associated with the development of inflammatory bowel disease and juvenile autism [Wakefield et al., 1993, 1997, 1998; Thompson et al., 1995]. Clinical samples from cases of Crohn's disease, ulcerative colitis, nondeterminate colitis, autistic enterocolitis, and subacute sclerosing panencephalitis (SSPE) have been examined in various laboratories for the presence of measles virus genome sequences [Iizuka et al., 1995; Haga et al., 1996; Chadwick et al., 1998; Afzal et al., 1998, 2000; Kawashima et al., 2000; Uhlmann et al., 2002]. Most laboratories have failed to demonstrate the presence of measles virus in clinical samples of inflammatory bowel disease patients [Iizuka et al., 1995; Haga et al., 1996; Chadwick et al., 1998; Afzal et al., 1998, 2000]; Two laboratories, however, have reported the detection of measles virus by either conventional RT-PCR [Kawashima et al., 2000] or TaqMan PCR [Martin et al., 2002; Uhlmann et al., 2002]. One explanation for the discrepant findings could be differences in sensitivity of the assays, which vary in the technical details between laboratories. The study described below was therefore designed to compare measles virus-specific RT-PCR-based methods established in various laboratories.

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MATERIALS AND METHODS

The participant laboratories that completed the study and returned results are shown in Table I.

Clinical Samples

Surgically removed portions of macroscopically inflamed areas of the gut of four Crohn's disease patients were obtained from Dr. S. Ghosh, University of Edinburgh, Scotland, UK. Their status with respect to the presence or absence of measles virus was unknown. An additional four sets of paired samples from Crohn's patients were produced in which one partner of the pair was spiked with measles virus and its corresponding partner was not spiked with any exogenous material. All clinical specimens used to produce the paired sample sets had been tested previously for measles virus and were found negative [Afzal et al., 1998, 2000]. Two were lymphocytes and two were specimens of intestine.

Measles Virus Strain

The wild-type measles virus strain, 94/31825 (UK 64), and a preparation of the SSPE brain suspension used in previous studies [Afzal et al., 1998, 2000] were used in this study. The SSPE strain, 96/33839, belonged to genotype A [Jin et al., 2002], whereas the wild-type strain, 94/31825, (GenBank accession numbers U29319 for M gene sequence and U29320 for N gene sequence) belonged to the genotype C2.

Spiking of Clinical Specimens With Measles Virus Strains

Portions of resected gut and lymphocyte preparations were spiked with 210 plaque forming units (pfu) of wild-type strain or with a fixed amount (10 μl) of the SSPE brain suspension as reported in Tables II and III. One sample (R) was provided as pre-extracted measles virus RNA derived from the wild-type strain and distributed in a final volume of 50 μl suspension that should have

TABLE I. List of Study Participants and Their Affiliations

Lab designation	Establishment	Contact person(s)	Address
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TABLE II. Detail of Clinical Materails Supplied

Code used	Clinical samples	Previous diagnosis of the clinical material in relation to measles virus persistence	Measles virus spiking	Total amount of measles virus in material distributed
A	Resected gut of a CD patient	Unknown, new patient	—	—
B	Resected gut of a CD patient	Unknown, new patient	—	—
C	Resected gut of a CD patient	Unknown, new patient	—	—
D	Resected gut of a CD patient	Unknown, new patient	—	—
E	Resected gut of a CD patient	Negative, patient -46 [Afzal et al., 2000]	SSPE brain material	?
F	Resected gut of a CD patient	Negative, patient -46 [Afzal et al., 2000]	—	—
G	Resected gut of a CD patient	Negative, patient -46 [Afzal et al., 2000]	Wild type strain	210 pfu
H	Resected gut of a CD patient	Negative, patient -46 [Afzal et al., 2000]	—	—
J	Lymphocytes of a CD patient	Negative, patient -4 [Afzal et al., 1998]	SSPE brain material	?
K	Lymphocytes of a CD patient	Negative, patient -4 [Afzal et al., 1998]	—	—
L	Lymphocytes of a CD patient	Negative, patient -4 [Afzal et al., 1998]	Wild type strain	210 pfu
M	Lymphocytes of a CD patient	Negative, patient -9 [Afzal et al., 1998]	—	—
R	Lymphocytes of a CD patient	Negative, patient -9 [Afzal et al., 1998]	Wild type strain	6.7 pfu (eqv).

contained measles virus RNA templates corresponding to 6.7 pfu. All samples were processed identically, aliquoted and stored at −70°C until transported on dry ice to all concerned laboratories.

Nucleic Acid Extraction and RT-PCR Amplification

All participating laboratories were requested to use their in house method(s) of nucleic acid extraction and PCR amplification. The procedures used are listed in Table IV. Laboratories used different RNA extraction procedures, and targeted different regions and sizes of the measles virus genome during RT-PCR or nested-PCR amplifications. More technical details of the procedures applied in different laboratories can be obtained from the contact person reported in Table I.

RESULTS

Originally, 13 laboratories that had an established record of measles virus RT-PCR work were invited to participate in the study. Of these 13 laboratories, four did not show interest for the study, one received the samples but did not complete the study and one responded positively in the beginning but failed to cooperate later. In total, seven laboratories completed the study successfully. The results of RT-PCR or nested PCR assays reported by various laboratories are summarised in Table III. Despite technical differences between the assays, the findings were mostly in agreement. Of the samples supplied, no laboratory detected measles virus sequence in any of the Crohn's disease patients, with the exception of Laboratory 5, which reported an ambiguous result for Sample A. All laboratories reported the presence of measles virus nucleic acid in all four samples from Crohn's disease patients spiked with measles virus or SSPE, as well as in the sample that contained pre-extracted measles virus RNA with the exception of Laboratory 7. Laboratory 2 reported an equivocal result for the SSPE-spiked sample E. Laboratory 7 failed to amplify the measles virus nucleic acid in two of the four spiked samples and from the sample containing pre-extracted measles virus RNA. Laboratory 3 reported the loss of RNA pellet of Sample H, which was the negative unspiked control of measles virus-spiked Sample G. Other samples from Crohn's disease patients, such as oral fluid, urine, or sera were not examined.

TABLE III. Summary of RT-PCR Results of Paticipating Laboratories

Sample code	Sample description	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
A	CD	−	−	−	−	+/−	−	−
B	CD	−	−	−	−	−	−	−
C	CD	−	−	−	−	−	−	−
D	CD	−	−	−	−	−	−	−
E	CD + SSPE	+	+/−	+	+	+	+	+
F	CD	−	−	−	−	−	−	−
G	CD + measles virus	+	+	+	+	+	+	−
H	CD	−	−	? RNA LOST	−	−	−	−
J	LYM + SSPE	+	+	+	+	+	+	−
K	LYM	−	−	−	−	−	−	−
L	LYM + measles virus	+	+	+	+	+	+	+
M	LYM	−	−	−	−	−	−	−
R	Measles virus RNA	+	+	+	+	+	+	−

TABLE IV. Comparative Evaluation of the Sensitivity Profile of Measles Virus Specific RT-PCR Assays Applied in Different Laboratories

Lab	RNA extraction method	RT-PCR	Nested PCR	Gene(s) targeted	Assay sensitivity in relation to pre-extracted measles virus RNA (sample R)	Assay sensitivity in relation to spiked measles virus particles	DNA product analysis	Related reference
1	RNAgents kit, Promega	rTth RT-PCR	Yes	N	1.34×10^{-3} pfu	2.1×10^{-4} pfu (based on sample G)	Ethidium bromide staining	Afzal et al., 1998, 2000
2	Promega "SV total RNA isolation system" kit (for solid samples & Roche "High pure viral RNA" kit (for suspension samples)	Con.RT-PCR	No	N	1.34×10^{-4} pfu	5.25×10^{-3} pfu (based on sample G)	Southern blotting using ^{32}P-labelled probe	El Mubarak et al., 2000; Osterhaus et al., 1997
3	Promega "SV total RNA isolation system" kit	Con.RT-PCR	Yes	P, F	1.34×10^{-3} pfu	2.1×10^{-1} pfu (based on sample G)	Ethidium bromide staining	Not supplied
4	Magnapure Automatic Extractor (Roche)	Con.RT-PCR	Yes	M, H	1.07×10^{-4} (based on M); 1.07×10^{-3} (based on H)	1.68×10^{-3} pfu (based on sample G)	Ethidium bromide staining	Jin et al., 1996, 1998
5	Qiagen RNeasy Kit	Con.RT-PCR	Yes	F	8.10×10^{-1} pfu	2.5×10^{-1} pfu (based on FDA Edmonston strain)	Ethidium bromide staining	Audet et al., 2000
6	Qiagen RNeasy Kit	Con.RT-PCR	No	P	3.35×10^{-2} pfu	1.05×10^{-3} pfu (based on sample L)	Ethidium bromide staining	Not supplied
7	Acid guanidinium thiocyanate phenol-chloroform method [Chomczynski and Sacchi, 1987]	Con.RT-PCR	Yes	H	All dilutions negative	2.1×10^{-1} pfu (based on sample G)	Ethidium bromide staining	Kawashima et al., 1996a, 1996b, 2000

Assay Sensitivity Evaluation

To evaluate the sensitivities of the assays applied for RT-PCR-nested PCR amplifications of measles virus RNA, participating laboratories were asked to determine cut-off values by assaying a tenfold dilution series of one measles virus spiked sample and a similar dilution series of pre-extracted measles virus RNA templates. The assay endpoints established by participating laboratories are shown in Table IV. Assay sensitivities, as determined with the pre-extracted measles virus RNA templates, ranged from 8.1×10^{-1} to 1.34×10^{-4} pfu between different laboratories. Laboratory 7 failed to detect any signal with measles virus pre-extracted RNA templates, even when they were assayed undiluted. It could be argued that samples supplied to the Laboratory 7 may have been degraded due to long distance shipment, but another laboratory from Japan was able to detect the measles virus sequence successfully.

The range of sensitivity between laboratories with pre-extracted RNA was about 1,000-fold. This does not take the results of Laboratory 7 into account.

The assay cut-off values established with RNA templates extracted in house from measles virus-spiked samples ranged from 2.1×10^{-1} to 2.1×10^{-4} pfu. The range of sensitivity between laboratories was again about 1,000-fold.

DISCUSSION

The key objective of this study was to compare RT-PCR methods developed by various laboratories with respect to the assay sensitivity, specificity, and reproducibility. Six of seven laboratories that completed the study detected measles virus nucleic acid in all measles virus-spiked samples including the sample that contained pre-extracted measles virus RNA templates. Laboratory 7 identified measles virus signals in less than 50% of known positive samples. It seems that most laboratories, irrespective of assay design and RT-PCR approach, would perform reasonably well in detecting measles virus by RT-PCR where its abundance is high. As seen in the sensitivity profiles of assays (Table IV), however, if the target templates are less abundant the laboratories would perform differently. The assays differ in sensitivity; laboratories that reported higher assay sensitivities using pre-extracted measles virus RNA templates also reported higher sensitivities with RNA templates extracted in house from the measles virus-spiked sample(s). This suggests that variation in sensitivity is not wholly attributable to variation in the nucleic acid extraction procedures used, but includes RT-PCR conditions themselves including the fragment size, oligonucleotide primer composition and sequence, sample consistency, and other experimental and technological parameters. As various laboratories targeted different regions of the genome, it is not evident that the primer sets used had homologous sequences to the target templates. The results of Laboratory 7 were inconsistent and lacked reproducibility, as it only identified measles virus RNA positively in one of two samples spiked with the same amount of measles virus, and one of two samples spiked with the same amount of SSPE tissue. The assay applied in this laboratory was about 1,000-fold less sensitive than the assay applied in the laboratory that performed best in this study. Further analysis of Table IV indicates that if the supplied materials had fewer than 10^{-3} virus particles, about 50% of the laboratories would have failed to identify them successfully. Therefore, it is vital that the assays used be of the highest sensitivity. Although none of the participating laboratories reported the presence of measles virus nucleic acid in any of the gut samples (A–D) that were derived from four new cases of Crohn's disease, laboratory 5 described an ambiguous result for one of these samples. It is reasonable to speculate that the measles virus signal observed in Sample A originated through cross contamination. Laboratory 5 had the least sensitive assay compared to other collaborating laboratories and it is unlikely that a positive result would have been missed by more sensitive protocols. The precise genetic identity of the finished DNA product of this sample in relation to its homology with measles virus strains is currently under investigation. Five of seven laboratories used a nested PCR, which indeed increases the sensitivity of the assay but also could increase the risks of laboratory contamination. From the results of Laboratory 2, it seems that the assay sensitivity could be improved without carrying out nested PCR, if the endpoint DNA products of RT-PCR amplification are assayed using an advanced detection procedure, such as hybridisation with an amplicon-specific probe. With the commercial availability of nonradioactive hybridisation reagents, it is probably reasonable to support the application of "amplicon-specific probe hybridisation approach" in all diagnostic RT-PCR assays to improve the sensitivity as well as to demonstrate the specificity of the target template amplified.

The present study lacks a direct comparison of conventional RT-PCR methods with TaqMan PCR used recently by Prof. J. O'Leary and associates in Dublin to detect measles virus nucleic acid fragments in the gut of autistic enterocolitis cases [Martin et al., 2002; Uhlmann et al., 2002]. The Dublin group also was invited to participate in this study but unfortunately did not take part.

In summary, this study highlights the following points: (1) laboratories may differ in sensitivity by as much as 1,000-fold from each other in terms of the ability to detect measles virus-specific sequences; (2) appropriate measures to prevent and identify contamination of samples with measles sequences are essential; (3) there is a need to establish a correlation between measles virus-specific conventional RT-PCR methods and TaqMan PCR to validate results reported by laboratories that applied one or the other method; (4) to avoid discrepancies between the laboratories it is vital that a correlation be established between the results described either in plaque forming unit or genome copy number; (5) collaborative studies are

probably the only way forward by which the presence or absence of measles virus in the gut and other tissues of inflammatory bowel disease and autism cases can be demonstrated effectively.

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