Rubella Virus RNA Assays:

Rubella Diagnostic RT-PCR Kit (conventional RT-PCR)

Rubella Virus Genotyping Kit (conventional RT-PCR, 2 Fragments)

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Basic Nomenclature for Wild-Type Rubella Viruses

Genetic characterization has identified 2 clades which differ by 8-10% at the nucleotide level. Clade 1 is divided into 10 genotypes (1a, 1B, 1C, 1D, 1E, 1F, 1G, 1h, 1i, and 1j), of which 6 are recognized and 4 are provisional (designated by lower case letters). Clade 2 contains 3 genotypes (2A, 2B, and 2C).

A Method for Detection of Rubella virus RNA by conventional RT-PCR

• Starts with a two primer set from Bosma, et al. in Journal of Clinical Microbiology, 1995.
• Produces a 185 nucleotide amplicon in the E1 protein coding region.
• Sensitivity has been shown to be approximately 100 copies of RNA.
RV12 Primer and Modifications

- The primer RV12 binds to a region of the genome where there is variation, especially between Clade 1 and Clade 2 viruses.
- RV12: CCA CAA GCC GCG AGT CA
- RV12-2: CCA CGA GCC GCG AAC AGT CG

A modified RV12 primer was made containing the Clade 2 majority sequence.

Rubella Virus Synthetic RNA Positive Control Construct
Diagnostic RT-PCR Kit

Current 3 Primer Conventional PCR for Rubella Virus RNA Detection

1 – Ladder
2 – Mock RNA
3 – Detection fragment
4 – Genotyping Fragment 1
5 – Genotyping Fragment 2
6 – Positive control (detection primers)

Lanes 3-5 contain products made with wild-type rubella RNA.
Lane 6 contains a product made using synthetic RNA with a 30 bp insertion.

Comparison of RT-PCR, serum IgM by 2 tests and of IgM

Challenges for sequencing rubella viruses directly from sample

- High GC content: kit selection
- Amplicon/sequence window size
- Primer design: sequence conservation
- Copy number in clinical samples
GC Content
- Rubella virus has a high GC content
  - 69.6% for the whole genome
  - 73% for the C coding region
  - 71% for the E2 coding region
  - 66.5% for the E1 coding region
- High GC content RNAs often contain stable intrinsic secondary structures that can inhibit reverse transcriptase and/or primer annealing
- Q Solution

Amplicon/Sequence Window Size
- Rubella sequence window is 739 nts in the E1 coding region (nts 8731-9469)
- RT-PCR amplicon needs to be larger (e.g. 945 nts) to allow for primer binding sites and low quality sequence close to the primer sites
- In general, the larger the amplicon, the higher the minimum copy number required for template production

Objective: Design a 2 Fragment System to Increase Sensitivity

2 Fragment System for Rubella Genotyping: Qiagen Kit Sensitivity
Template is F-Therien RNA, 1X10^6 – 1X10^7 copies

Qiagen RT-PCRs on rubella viruses with changes in at least 1 primer binding site
1: 8633/9112 8 viruses
2: 8945/9577 6 viruses

All reactions were positive for both fragment 1 and fragment 2.
Rubella RNA copy numbers in 29 clinical samples

<table>
<thead>
<tr>
<th>Place</th>
<th>Genotype</th>
<th>Sample type</th>
<th>Day of coll</th>
<th>Copy #/2.5ul</th>
<th>Single (945nt)</th>
<th>Frag 1 (480nt)</th>
<th>Frag 2 (633nt)</th>
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<td>MI</td>
<td>2B TS</td>
<td>&gt;10</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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<td>&gt;10</td>
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</tr>
<tr>
<td>Peru</td>
<td>1C oral fluid</td>
<td>0</td>
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<td>nd</td>
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<td>Positive</td>
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<tr>
<td>Yemen</td>
<td>2B swab</td>
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<td>MN</td>
<td>2B NP</td>
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<td>CA</td>
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<td>3 mo (CRS)</td>
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<tr>
<td>Peru</td>
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<td>0</td>
<td>267</td>
<td>nd</td>
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<td>Peru</td>
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<td>Positive</td>
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<td>nd</td>
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<td>AZ</td>
<td>1G NP-1</td>
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<td>4536</td>
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<tr>
<td>ME</td>
<td>1E TS</td>
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<td>50,000</td>
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<td>Yemen</td>
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<td>8812</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

20% were positive for single amplicon
100% over 100 copies were positive for both smaller amplicons

Rubella Genotyping Procedure

• Extract RNA from throat swab, oral fluid, or urine using Qiagen Viral RNA kit or equivalent
• Screen for rubella RNA using real-time RT-PCR or high sensitivity conventional RT-PCR (e.g. RV11 RV12 RV12-2): 185 bp amplicon

Rubella Genotyping Procedure, cont.

• If either real-time or conventional RT-PCR is positive, set up 2 conventional RT-PCR reactions using primers 8633/9112 (480 bp) and 8945/9577 (633 bp).
• Analyze the products on a gel and, if positive, clean the RT-PCR reactions and perform sequencing reactions.

Rubella Virus Synthetic RNA Positive Control Constructs

Works with both 3' and 5' fragments.

Two Fragments for Rubella Genotyping

The positive control (PC) contains a 30 nt insertion in fragment 1 and an 84 nt deletion in fragment 2.

Table 1. Global Distribution of Reported Rubella Genotypes, 2005-2010

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of reports</th>
</tr>
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<tr>
<td>1a</td>
<td>17</td>
</tr>
<tr>
<td>1B</td>
<td>2</td>
</tr>
<tr>
<td>1C</td>
<td>3</td>
</tr>
<tr>
<td>1E</td>
<td>2</td>
</tr>
<tr>
<td>1G</td>
<td>26</td>
</tr>
<tr>
<td>1h</td>
<td>2</td>
</tr>
<tr>
<td>1j</td>
<td>2</td>
</tr>
<tr>
<td>2B</td>
<td>5</td>
</tr>
<tr>
<td>2C</td>
<td>9</td>
</tr>
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</table>

* Genotypes 1D, 1F, 1i, and 2A were inactive during this period.
* Country(ies) and year(s) of report are indicated.
* Probable import, but links unknown.
Rubella virus genotypes reported sporadically or in geographically restricted regions. (Probable imports with unknown links not shown)

Sample processing for RNA extraction and virus isolation

- **Extract RNA**
  - Advantage: quick (1-2 days)
  - Disadvantages: limited amount of RNA
  - RT-PCR and sequencing can be difficult.

- **Infect cells**
  - Disadvantage: slow (1-3 weeks)
  - Advantages: unlimited amounts of RNA
  - RT-PCR and sequencing is usually easy.

- **Store at -70 °C**

- **140 µl**
- **0.5 ml**
- **remainder**

Acknowledgements

CDC MMRHLB

- Emily Abernathy
- Dr. Min-hsin Chen
- Dr. Ludmila Perelygina
- Ada Ogee-Nwanko
- Lijuan Hao

Rubella Virus RNA Assays:

Rubella Diagnostic RT-PCR Kit (conventional RT-PCR)

Rubella Virus Genotyping Kit (conventional RT-PCR, 2 Fragments)

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Protocol information—-Monday

A. Rubella Virus Genotyping Kit
(We learned in preparing for this course that dried control RNA has instability in the Fragment 1 (5', c-myc) region. (We are working on this!!!)

i. So, we will use the Rubella Diagnostic RT-PCR Kit control for Fragment 1

ii. Positive control

1. Use 1 ul of positive control of master stock from Diagnostic RT-PCR kit

3. Fragment 2

4. Resuspend Rubella Virus Genotyping Kit positive control in 100 ul of nuclease free-water.

5. Use 1 ul as positive control for rubella virus genotyping kit.

6. NOTE: in your home labs, you should made 10 ul aliquots and store at – 70 C. Remove 1ul from one of these aliquots.

ii. Wild-type rubella virus

1. Use 5 ul of redissolved RNA (made in Rubella Diagnostic RT-PCR kit procedure).

Exercises 2a and 2b

1. Templates and Volumes to use

a. Rubella Diagnostic RT-PCR Kit

i. Positive control

1. Resuspend in 50 ul nuclease-free water (master stock), dilute 5 ul into 50 ul nuclease free water (1/10) working stock

2. Use 1 ul of working stock as positive control in reactions

a. 5 ul aliquots of master stock are stored at -70 and working stock is only frozen and thawed 5 times.

ii. Wild-type rubella virus (Control from CDC)

1. Resuspend dried RNA as described on tubes in 1 ml nuclease free water

2. Use 5 ul as wild-type rubella in reactions

We will carry through the RNA from rubella virus infected cells and control

1. Templates volumes and identity are:

a. Diagnostic

i. 5 ul negative control extracted RNA

ii. 5 ul virus positive extracted RNA

iii. 5 ul control RNA (wt-rubella RNA) from CDC

iv. 1 ul Diagnostic Positive control from CDC (insert)

+ 4 ul nuclease free water

b. Sequencing Fragment 1

i. 5 ul negative control extracted RNA

ii. 5 ul virus positive extracted RNA

iii. 5 ul control RNA (wt-rubella RNA) from CDC

iv. 1 ul of master stock from Diagnostic RT-PCR kit

+ 4 ul nuclease free water

c. Sequencing Fragment 2

i. 5 ul negative control extracted RNA

ii. 5 ul virus positive extracted RNA

iii. 5 ul control RNA (wt-rubella RNA) from CDC

iv. 1 ul of Positive Control RNA from Sequencing kit

+ 4 ul nuclease free water
Updates of WPRO measles and rubella laboratory network

Youngmee Jee (M.D. Ph.D.)
Expanded Programme on Immunization
Western Pacific Regional Office
World Health Organization

Outline

- Regional EPI goals
- Measles and rubella incidence and vaccination, challenges and plans
- Achievements and Monitoring of performances of WPR measles labnet
- Genotyping data in WPR
- Training, meetings and plans
- Objectives of the training

Regional goals

- 2003: RC resolution WPR/RC54.R3: established regional goals of measles elimination, Hep B control and maintaining polio-free status
  - Urged Member States to "use measles elimination and hepatitis B control strategies to strengthen EPI and other public health programmes, such as prevention of congenital rubella syndrome"
- 2005: RC resolution WPR/RC56.R8 established 2012 as the target date to achieve the "twin goals": agreed by all member states
  - Achieve measles elimination
  - Reduce chronic hepatitis B infection rates to < 2% in 5 year old children as an interim milestone towards final goal of < 1%
- 2010: to achieve and maintain control of rubella and prevention of congenital Rubella Syndrome in the WPR by 2015 (19th TAG)
  - Rubella: ≤ 10 / 1 million population, excluding imported cases
  - CRS: ≤ 10 / 1 million LBs, excluding imported cases

Measles Elimination Progress

Measles Drops 58% in the Western Pacific Region!

Measles Down an Incredible 93% Two Years after Japan Launches Its Measles Elimination Plan!

China's SIA Intensification Yields a Decrease of 60% in Measles Cases and 64% in Deaths! Nationwide SIA in 2010

25 Countries and Areas in the Western Pacific May Already Have Eliminated Measles!

WHO Announces Regional Verification Commission for Measles Elimination to be Formed!
Technical Consultation on Verification of Measles Elimination

Measles Incidence*

Western Pacific Region, 2009

* per million population

Measles Cases, by Month, China, 2005-2010*

*Data as of 19 August 2010
**Comparison in number of cases in January-July 2010 with the same period 2009

Incidence rates (per million)

- 2005: 104.0
- 2006: 81.4
- 2007: 88.8
- 2008: 98.4
- 2009: 39.0
- 2010: 60% 18%**
### Challenges to Measles Elimination

- Coverage and surveillance performance is not homogenously high across the countries and within countries
  - Low sensitivity of surveillance at sub-national levels
  - Accumulation of susceptibles
  - Large proportion of clinically confirmed cases in some countries
- Obtaining accurate epidemiologic data
  - Incomplete case investigation data
  - Discrepancies between lab reports and national reports
- Changing epidemiology and unexpected outbreaks
  - Age distribution shifting to infants and adults
  - Increased risk in densely populated areas
- Importations within the WPR and from other regions, floating populations
- Resource mobilization (funding for SIAs) and political commitment
- Monitoring genotype changes
- Complexity of case classification

### Plans for Measles Elimination in the Western Pacific Region by 2012

- SIAs in CHN, PHL, PNG, VTN in 2010; CAM & LAO in 2011
- Optimize routine schedules and approaches to minimize immunity gaps and maximize coverage
- Strengthen epidemiologic and laboratory surveillance and communication
- Engage partners and political leaders
- Establish regional and national verification committees for measles elimination by early 2011
- Advocacy for high level political commitment to mobilize human and financial resources

### EPI Laboratory Network in WPR

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<thead>
<tr>
<th>Type of Vaccine</th>
<th>Polio</th>
<th>Measles/rubella</th>
<th>IPV</th>
<th>Rota</th>
<th>HPV</th>
<th>HBV</th>
<th>IBD</th>
<th>Total No.</th>
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<td>2 NSIL, Japan</td>
<td>1 - RIVM, Japan</td>
<td>1 NSIL, Japan</td>
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<td>4</td>
<td>0</td>
<td>7</td>
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<td>2 - VIML, Australia, - CDC, China</td>
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<td>6</td>
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<td>National</td>
<td>0</td>
<td>56 including 412 labs in 16 FL, EP, NC, Guam</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>61</td>
<td></td>
<td></td>
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<tr>
<td>Sub National</td>
<td>31 provincial laboratories in China</td>
<td>31 provincial = 331 prefectural laboratories in China</td>
<td>Some provincial labs in CHN(7)</td>
<td>331</td>
<td></td>
<td></td>
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<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>46</td>
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</table>

### WPR Measles and Rubella Laboratory Network

- 382 laboratories
  - 331 provincial
  - 16 national (13 fully functional)
  - 25 prefectural labs

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*Data for January – August 2010*
Data for January – October 2010, as of Nov 16 2010

Deadline for submission is on the 10th of the following month.

Regional Laboratory Training/Hands-on workshop in October 2009 for priority countries: ELISA/DBS, virus culture, molecular detection.

Financial support to cover operational costs (TSA) and kits and equipment support for the NMLs in priority countries from 2009.

China labnet:
- implemented excellent QA programme including PT and confirmatory testing for prefrectual labs as well as for provincial labs; WHO global PT samples to 31 provincial labs from 2009 and all scored 100%.
- Most provincial labs perform virus isolation using Vero/SLAM cells and RT-PCR.
- In 2009-2010, all provincial labs implemented real time PCR for measles, rubella and mumps.
- Annual meetings and hands on training courses for 31 provincial labs organized by China CDC.
- Tibet has been on site reviewed in 2010 for the first time in 2010.

2006-2010:
- 2006: 12/14 (85.71%)
- 2007: 10/15 (60%)
- 2008: 35/37 (90%)
- 2009: 37/37 (100%)
- 2010: 60/60 (100%)

All 49 laboratories receiving WHO global PT samples passed WHO proficiency test. Most lab scored 100% (100% for 16/18+31/31=47/49 labs, 95% two labs for both measles and rubella).

Genotyping data on recent measles virus strains available from most countries except PNG, Fiji and other PICs.

2006: D9(2)
2007: D9
2008: D9
2009: D9
2010: D9

Complications and Timeliness of Measles Lab Reporting Western Pacific Region, 2008 – 2010*

- Measles IgM
- Measles IgG
- Rubella IgM
- Rubella IgG

WPR Measles/rubella LabNet Updates

- Accreditation status: All GSL and RRLs, 10/13 national labs including Fiji excluding three PIC labs, all 31 provincial labs in China are accredited as of Nov 22, 2010.
- Confirmatory testing in all NMLs: twice a year from 2010, concordance rates in 2009: >90%-100%. All 49 laboratories receiving WHO global PT samples passed WHO proficiency test. Most lab scored 100% (100% for 16/18+31/31=47/49 labs, 95% two labs for both measles and rubella).
- Genotyping data on recent measles virus strains available from most countries except PNG, Fiji and other PICs.

2006: 3/4 (75%)
2007: 7/7 (100%)
2008: 8/15 (53.34%)
2009: 14/15 (93.3%)
2010: 8/8 (100%)

2006: 4/6 (66.67%)
2007: 8/10 (80%)
2008: 15/15 (100%)
2009: 10/10 (100%)
2010: 10/10 (100%)

2006: 19/19 (100%)
2007: 10/10 (100%)
2008: 102/110 (92.72%)
2009: 10/10 (100%)
2010: 80/80 (100%)

2006: 11/15 (73.33%)
2007: 7/7 (100%)
2008: 42/51 (82.35%)
2009: 44/45 (97.78%)
2010: 48/50 (96%)

2006: 12/15 (80%)
2007: 39/45 (86.67%)
2008: 62/70 (88.57%)
2009: 72/72 (100%)
2010: 60/60 (100%)

2006: 14/20 (70%)
2007: 32/48 (66.67%)
2008: 57/60 (95%)
2009: 62/65 (97%)
2010: 48/50 (96%)

2006: 7/8 (87.5%)
2007: 8/10 (80%)
2008: 7/8 (87.5%)
2009: 10/10 (100%)
2010: 17/20 (85%)

2006: 20/22 (95.23%)
2007: 56/57 (98.24%)
2008: 20/22 (95.23%)
2009: 60/60 (100%)
2010: 60/60 (100%)

2006: 3/4 (75%)
2007: 7/7 (100%)
2008: 8/15 (53.34%)
2009: 14/15 (93.3%)
2010: 8/8 (100%)

2006: 4/6 (66.67%)
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2006: 19/19 (100%)
2007: 10/10 (100%)
2008: 102/110 (92.72%)
2009: 10/10 (100%)
2010: 80/80 (100%)

WPR measles/rubella LabNet

Updates

IgM testing Workload (except China and Japan) based on lab reports: 2006-2010*
Summary of Virologic Surveillance and genotyping of Measles in WPR

- Recent genotype information is available for most countries in the region and HK, China, Singapore, Australia and Japan share genotype information with WHO. Genotype information for other countries (Cambodia, Laos, Mongolia, Philippines, Vietnam) reported by Hong Kong RRL.
- Many national and China sub-national labs as well as RRL, GSL have laboratory capacities to perform PCR, sequence analysis and virus isolation.
- HK RRL conducted genotyping/sequencing of measles and rubella viruses using confirmatory serum samples for Vietnam, Cambodia, Laos, Philippines, Mongolia, Malaysia and Macao.
- Endemic transmission of H1 strains in China and Vietnam
- D9 strains detected in many countries including Malaysia, Singapore, Philippines, Cambodia and Laos in 2009-2010.

3rd Regional Hands on training for measles and rubella labs in Oct 2009

- Laboratory Hands on training course in HK China in October 2009 for priority countries:
  - Participants from China, Cambodia, Laos, Malaysia, Mongolia, Philippines, Vietnam, Fiji
  - ELISA/DBS, cell culture, molecular detection of measles and rubella viruses
  - Facilitators from US CDC, NIID and WHO.

Measles Rubella Session during 2nd VPD Labnet meeting

- Two day meeting in Feb 25-26 2010
- All NML, RRL, GSL attended: participants from 17 network laboratories and National surveillance/EPI officers
- Discussed how to strengthen the quality of the performances of measles/rubella laboratory networks and to enhance the regional capacity for virus isolation and genotyping.
Recommendations from 2nd Labnet meeting, 2010 Feb

3) Molecular surveillance
As recommended by the Global Measles Laboratory Network Meetings (Sep 2008 and Oct 2009), laboratories are encouraged to submit representative genotype/sequence information on their measles and rubella viruses to the WHO genotype and MeaNS databases, preferably on a "real-time" basis, but at least by the end of the month in which the genotyping was completed. A copy of the information should be sent also to the regional laboratory coordinator.

a. Laboratories which participated in the regional hands on laboratory training in 2009 and other sequencing laboratories, are encouraged to perform virus isolation and/or molecular detection of measles and rubella viruses to identify the genotype and obtain sequence information on circulating measles and rubella viruses.

b. Efforts should be made to collect sequence data from all chains of infection, especially in those countries where no baseline sequence data exists.

c. The Labnet should utilize the well validated tools and samples available for enhancing molecular surveillance where appropriate, such as:

i. Oral fluid, throat swabs, urine and PBMC as samples for virus detection
ii. Detection of viral RNA in archival sera
iii. Standardized PCR methods including the use of a validated, unique PCR control.

Priority indicators for components of the verification process for measles elimination in WPR: Laboratory

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Core</th>
<th>Complementary</th>
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<tr>
<td>All WHO measles labs accredited</td>
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</tr>
<tr>
<td>&gt;80% cases confirmed at WHO accredited labs</td>
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</tr>
<tr>
<td>&gt;80% outbreaks with virologic and genotypic analysis</td>
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<tr>
<td>100% concurrence of lab-confirmed cases between surveillance and lab units</td>
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<tr>
<td>Baseline virologic (genotype) surveillance data available</td>
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<tr>
<td>Timely sharing laboratory testing data with WPRO and genotype data within 2 months with WPRO &amp; WHO HQ</td>
<td>✓</td>
<td>✓</td>
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</table>

Plans for Labnet in 2010-2011

- Building up further capacity for virologic surveillance in the region:
  - Follow up Hands on training in Nov 22-27, 2010
  - To discuss the regional data management
  - To enhance knowledge and skills in:
    - Improved completeness and timeliness (100%) of case based laboratory reporting and genotype reporting for measles/rubella viruses
    - Meeting training workshop

Objectives of training

- To enhance knowledge and skills in:
  (a) molecular detection and genotyping of measles and rubella viruses (RT-PCR and sequencing)
  (b) sequence analysis of measles and rubella viruses
- To discuss the regional data management using the new laboratory reporting format and sharing and reporting genotype and sequence data

Day 1, Monday

- Session 1: Lectures on Global and regional update on measles, rubella and molecular epidemiology
  Lunch
- Session 2: two practicals - 1) RNA extraction and 2) RT-PCR for rubella detection and genotyping

Acknowledgements

WHO HQ David Featherstone
US CDC Paul Rota
US CDC Joe Icenogle
Xu Wenbo and CCDC team
Wilina Lim and HK PHL team
WPRO EPI Measles team
WHO country EPI officers

Technical Consultation meeting for verifying measles elimination - June 2010

World Health Organization • Western Pacific Regional Office • Expanded Programme on Immunization
Day 2, Tuesday

- Session 3: Practicals
  1) RT-PCR for measles genotyping and
gel electrophoresis of rubella detection &
genotyping RT-PCR
  2) gel electrophoresis of rubella detection &
genotyping RT-PCR
  3) gel electrophoresis of RT-PCR for measles
genotyping
  4) PCR purification of RT-PCR for measles and
rubella genotyping
  5) gel electrophoresis of purified PCR products
(measles and rubella)

Country reports (China, Japan, Malaysia,
Mongolia, New Zealand) - Can be moved to
Thursday

Day 3, Wednesday

- Session 4: Practicals- Cycle sequencing,
purification of cycle sequencing products and
demonstration of sequencing run

Country reports (Philippines, Korea, Singapore,
Viet Nam NIHE and PI)

Day 4, Thursday

- Session 5: Practical - Sequence analysis
for measles virus genotyping

Country reports

Day 5, Friday

- Session 6: Data management and reporting
  - Monthly laboratory data to Western Pacific
    Regional Office (WPRO)
  - Genotype and sequence data submission to
    WHO HQ and Means

- Session 7: Practical: Sequence analysis for
rubella virus genotyping

Day 6, Saturday

- Course assessment and quiz
- Next steps, summary of assessment and quiz
- Distribution of MR IgM ELISA proficiency test
  panel samples (reporting within 14 days) and
  filter paper proficiency test panel (reporting
  within 2 months)
- USB with all presentation files and reference
  materials
- Closing and presentation of certificates

Measles outbreaks and
importations in 2009-2010

Vietnam, Philippines
New Zealand, Australia....
Genotyping Results of Measles viruses in 2009: New Zealand

<table>
<thead>
<tr>
<th>Sample</th>
<th>Virus No.</th>
<th>Date</th>
<th>Genotype</th>
<th>Sequence run</th>
<th>Comments</th>
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<td>D4</td>
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<td>D4</td>
<td>5/11/2009</td>
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<td>D4</td>
<td>5/11/2009</td>
<td>Genotype result</td>
</tr>
</tbody>
</table>

103 measles IgG positive cases in 2009, 10 genotyped

Courtesy: Dr. Anjali Wahi and New Zealand Ministry of Health

Measles Virus Importation, Australia 2008-2009

- Virus has entered Australia from at least 13 countries in all 6 Regions
- India - 104 cases = 4.9/million
- Australia - 70 cases = 3.3/million
- Import-related cases

Measles Cases, Australia 2009*

- 104 cases = 4.5/million
- 24 importations from 13 countries in 6 WHO Regions
- 70 cases = 3.3/million
- 8 sporadic cases = 0.4/million

Measles Cases, by Month of Rash Onset and Method of Confirmation, Cambodia 2003 – 2010*

- Expected suspected measles cases: 295
- No. AFR cases reported: 4,779 in 2010

Atypical Measles outbreak (1T) among students with high vaccination coverage in one middle school in 2010 in Incheon, Korea

- 69 vaccinated out of 71 lab confirmed cases
- First generation of spread
- Second generation of spread
- Third generation of spread
- First case reported to KDCA
- KDCA interventions begin

Unpublished Data from Korea CDC
Progress Towards Measles Elimination
Western Pacific Region 2007-2010*
Power of molecular surveillance

- Sequence information, in combination with epidemiological data can:
  - Allow mapping of transmission pathways
  - Identify possible source of virus and determine whether it is indigenous or from an imported source
  - Determine whether suspected case due to vaccine or wild virus
  - Assist with confirmation of true positives
  - Improve diagnostic resolution in first few days after rash onset in combination with IgM

Identifying the geographical origin and tracing the transmission pathways of a virus

- Building comprehensive knowledge of global distribution of virus molecular data
  - Source of virus in new outbreaks may be determined

- Sequence information alone may not be sufficient to identify source or transmission pathways
  - Epidemiological investigations can also identify possible source of origin and/or transmission pathways

- Combination of epidemiological and molecular data ideal
  - Outbreak investigation with comprehensive laboratory analysis

Capacity for Molecular testing increasing

- Molecular capacity has become accessible to more labs
  - National and even some sub-national labs have PCR equipm’t
  - Real-time and conventional PCR

- Specific training workshops focusing on molecular techniques held in all WHO regions

- Kit based RT-PCR systems increase ease of use

- Oral fluid samples may permit detection of measles and rubella RNA up to several weeks after disease onset

Indicators for determining Elimination*

DEFINITIONS:

- **Measles eradication.** Worldwide interruption of measles transmission in the presence of a verified, well-performing surveillance system.

- **Measles elimination.** The absence of endemic measles transmission in a defined geographical area (e.g. region) for a period of at least 12 months or more, in the presence of a well-performing surveillance system.

- **Endemic measles transmission.** The existence of continuous transmission of indigenous or imported measles virus that persists for a period of 12 months or more in any defined geographical area.

* WER 3 Dec 2010
Indicators for determining Elimination

- **Re-establishment of endemic transmission.** Re-establishment of endemic measles transmission is a situation in which epidemiological and laboratory evidence indicates the presence of a chain of transmission of a virus strain that continues uninterrupted for a period of twelve months or more in a defined geographical area where measles was previously eliminated.

- **Measles outbreak in countries with an elimination goal.** When two or more confirmed cases are temporally-related (with 7-21 days between dates of rash onset), and are epidemiologically and/or virologically linked.

- **A measles imported case.** A case exposed outside the region/country during the 7 to 21 days prior to rash onset, as supported by epidemiological and/or virological evidence.

2 Viruses with N gene (450) sequences that are at least 99.7% identical (1 nt change)

Rationale behind development of genotype/sequence databases

- Labs with sequence data not always aware of importance of sharing with surveillance programme
  - Data sometimes released months or years after sample collected
  - Data sometimes kept until publication finalized
- Countries and regions at different stages of control/elimination
  - Elimination countries need to know whether cases detected are due to imported or indigenous virus ASAP
  - Control countries need to know their baseline sequence data
- Centralised databases will allow ready access to all reported viruses

**Decision to develop database for Sequences/Genotypes**

<table>
<thead>
<tr>
<th>2006 WHO Genotype Database</th>
<th>2008 MeaNS Sequence Database (HPA)</th>
<th>GenBank</th>
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<tbody>
<tr>
<td>Genotype and epi data</td>
<td>Sequence and epi data</td>
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<tr>
<td>GenBank access No.</td>
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**Databases**

- **Decision for two databases at 2006 Global meeting:**
  - Genotype database WHO database – Simple, Excel based
  - Sequence database MeaNS

**Issues**

- Accessibility to sequence database
- Accessibility to non-public domain sequence data
- Frequency of updating data
  - More timely = more useful for programmatic application,
  - Preferably real time access

**Variables for WHO Genotype Database**

- ISO country code and country name
- Date of case: (month, year)
- WHO name of virus: e.g. MViiGuizhou.CHN/47.09/1[H1]
- Genotype
- Epi link (if known)
- Submission data (who, when)
- GenBank accession number – access to sequence data
WHO Global genotype database: Current Status

Viruses contributed to WHO database or GenBank

WHO Global genotype database: Current Status

WHO EMR PAK Pakistan 6 entries

WHO EMR SOM Somalia 37% with GenBank

WHO EMR DJI Djibouti 23 entries

WHO EMR KW T Kuwait 23 entries

WHO Measles Measles 8553 entries

WHO Rubella 749 entries

WHO Global genotype database: Current Status

Number of viruses Genotypes Countries WHO Regions Proportion with GenBank entries

Measles 8553 23 118 6 37%

Rubella 749 11 40 6 39%
**WHO Vaccine Preventable Disease Lab Network**

**WPR Country Submissions to Measles Databases**

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**Measles Viruses by Year of Detection 1999-2010**

As of Sept 2010

**Countries With No Measles Genotype Information Reported**

<table>
<thead>
<tr>
<th>Region</th>
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<tr>
<td>Others</td>
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</tbody>
</table>

**MeaNS Measles Sequence Database**

Data manually submitted or automatic downloads from GenBank
- N - 450 – 4900 sequences
- N – full – 5 sequence
- H – full – 486 sequences

Ability to sort if large number of identical hits

Mapping feature – not available/working
Timely submission of sequences to MeaNS allows all for all rubella virus genetic information, the WHO Vaccine Preventable Disease Lab Network

For all rubella virus genetic information, the WHO database, either through MeaNS or directly.

Submission to GenBank - 450 or 456 nt e-mail confirmation etc sent to you (not MeaNS)

MeaNS database

http://www.who-measles.org – live

Practice site – for meeting
http://www.hpa-bioinformatics.org.uk/gnani/Measles/Public/Web_Front/main.php

User name: labnet1......labnet10
Password: labnet99

Acknowledgments:
Richard Myers, S (Gnaneshan) Gnaneshan, Jon Green, David Brown.

All those who have submitted data

Recommendations from Global LabNet Meeting 2010

Timely submission of sequences to MeaNS allows all reporting criteria to be met in one action as these data are automatically submitted to the WHO database and optionally to GenBank. MeaNS also assists with characterization and QC of the virus sequence.

At a minimum, measles virus genotypes are to be submitted to the WHO database, either through MeaNS or directly.

For all rubella virus genetic information, the WHO database should be used with sequence data preferably also submitted to GenBank.

Summary & Recommendations

- Reporting of timely sequence information critical for programme monitoring and determining elimination
- Need to identify sequences from all outbreaks and chains of infection
- Timeliness of genetic information reporting monitored through Accreditation checklist
- Recommended that measles viruses are deposited in MeaNS database (with sequence)
- Rubella viruses should continue to be deposited in WHO database

Acknowledgements

- HPA and CDC
- All labs who have contributed timely data
- Youngmee Jee for following up on labs

Thank You!!
Day 1, 22 November

Grouping arrangement for practicals

- **Group A (China x 2)**
- **Group B (Philippines x 2)**
- **Group C (Vietnam x 2)**
- **Group D (Malaysia & Singapore)**
- **Group E (Korea & New Zealand)**
- **Group F (Japan & China)**
- **Group G (Mongolia & Hong Kong)**

**Practical: RNA extraction for measles and rubella**

- Each group will be given 5 samples
  - Measles: 2 urine samples and 1 culture fluid
  - Rubella: 1 urine sample and 1 culture fluid
- Qiagen QIAamp Viral RNA Mini kit
  - Reconstitute carrier RNA with buffer AVE (310µl)
  - Prepare buffer AVL by adding reconstituted carrier RNA (100µl reconstituted carrier RNA to 10ml buffer AVL-provided)
  - Absolute ethanol and buffers AW1 & AW2 (with ethanol added), 1.5ml snap cap vials (caps cut away by scissors) will be provided
- Participants will take turns to add samples to buffer AVL in safety cabinet (Rooms 906 & 907)-white gown
- Subsequent extraction steps will be performed in Room 907
- After elution, keep RNA on ice and go to Room 809
- Take off white gown before leaving Room 809

**Practical: Rubella detection & genotyping RT-PCR**

- Separate rooms will be used
  - Room 811: Master mix preparation (blue gown)
  - Room 809: RNA template addition (white gown)
  - Room 817: PCR product handling (yellow gown)
- Participants will take turns to prepare master mix in safety cabinet or PCR workstation in Room 811-blue gown
- Qiagen One-Step RT-PCR kit + RNase inhibitor (ABI)
- Primers in working concentration (20µM) will be provided
- 3 sets of PCR
  - Rubella detection: 3 primers (RV11, RV12 & RV12-2)
  - Rubella genotyping: 2 sets of primers (8633F & 8945F & 9577R)
  - RNA samples to be tested: 2 extracted RNA + 1 wild type RNA from CDC; all three add 5µl to PCR tubes

Day 2, 23 November

**Practical: Measles genotyping RT-PCR**

- Take turns to prepare master mix in Room 811-blue gown
- Qiagen One-Step RT-PCR kit + RNase inhibitor (ABI)
- Primers in working concentration (20µM) will be provided
  - Two primers (MeV216 and MeV214)
- Keep PCR master mix on ice
- Take off blue gown before leaving Room 811
- Put on white gown in Room 809
- Three rubella RNA controls will be provided for
  - Detection RT-PCR: add 1 µl to PCR tube
  - Genotyping fragment 1 RT-PCR: add 1 µl to PCR tube
  - Genotyping fragment 2 RT-PCR: add 1 µl to PCR tube
- Keep the PCR tubes on ice
- Carry the PCR tubes to Room 817
- Take off white gown before leaving Room 809
- Two groups will share one ABI 9700 thermal cycler
- Both detection and genotyping RT-PCR share the same PCR conditions
Practical: Gel electrophoresis for rubella RT-PCR
- 2% gels will be provided (one gel tank per group)
- 96-well plate for mixing samples and loading dye
- Samples: 2µl 6X loading dye + 5µl samples
- Marker: 2µl 6X loading dye + 2µl 123bp ladder + 8µl 1X TBE
- Load mixtures to the gel (Remember the sample order!)
- Power supply will be shared (140V, 25-30min)
- Avoid taking things into & out of Room 817
- Take off yellow gown and wash hands before leaving Room 817

Practical: Gel electrophoresis for measles RT-PCR
- Similar to gel electrophoresis for rubella RT-PCR

Practical: PCR purification for measles & rubella
- Qiagen QIAquick PCR Purification kit
  - Buffer PE (with ethanol added) and 1.5ml snap cap vials (caps cut away by scissors) will be provided
  - Purify all positive samples, including wild type RNA from CDC
- Put on yellow gown in Room 817
- Avoid taking things into & out of Room 817
- Take off yellow gown and wash hands before leaving Room 817

Practical: Gel electrophoresis of purified PCR products
- Similar to previous gel electrophoreses
- Determine amount of DNA templates to be added for cycle sequencing the next day

Day 3, 24 November
Practical: Cycle sequencing
- Prepare cycle sequencing master mix in Room 811
- BigDye Terminator v3.1, sequencing primers (3.2µM) and water will be provided
- Keep cycle sequencing mix on ice
- Add the sequencing primers in Room 817
- Two groups will share one ABI 9700 thermal cycler
- Avoid taking things into & out of Room 817

Practical: Purification of cycle sequencing products
- Qiagen DyeEx 2.0 Spin kit
- Put on yellow gown in Room 817
- Speedvac (~1hr)

Practical: Sequencing run
- Fill in sample ID in worksheet and computer of sequencers
- Add Hi-Di formamide in safety cabinet (Rooms 839 & 840)
- Separate sequencers will be used for measles and rubella
3. Add 1 µl of sequencing primer to each tube according to worksheet

4. Add 1 µl of corresponding purified PCR product to each tube
Introduction to Molecular Techniques for Detection of Measles Virus and Genotyping by RT-PCR and Real-Time RT-PCR

Paul A. Rota, Ph.D.
Measles, Mumps, Rubella and Herpes Viruses Laboratory Branch
Division of Viral Diseases,

Importance of Virologic Surveillance and Viral Detection
Molecular techniques can provide a valuable tool to:
• Differentiate between ongoing transmission of endemic virus from new, imported source of virus
• Aid in the classification of unusual or severe cases
• Confirm suspected vaccine reactions
• Molecular techniques have an increasing role in case confirmation especially in low incidence settings and/or when serologic results are difficult to interpret

Lessons Learned from Nearly 20 Years of Virologic Surveillance for Measles Virus-1
• Molecular epidemiologic studies are a key component of verification of measles elimination
  • One indicator for verification of elimination will be absence of an endemic genotype for one year
  • Genetic data can be used to track transmission patterns and identify sources of infection
  • There is rapid global transmission of measles viruses. For example, viruses associated with outbreaks in Africa are soon detected in association with imported cases in the Americas
  • It is impossible to predict how/when a virus will spread from an endemic area to other parts of the world
• Vaccination programs frequently interrupt transmission of measles lineages, but reintroduction of measles is a problem (sometimes with an apparent switch in genotype)

Lessons Learned from Nearly 20 Years of Virologic Surveillance for Measles Virus-2
• All measles vaccines are in genotype A
• Vaccination has been effective globally despite the presence of different endemic genotypes (provided the vaccine is administered properly)
• Molecular epidemiologic data clearly show that it is possible to maintain elimination of measles transmission despite constant importation of multiple genotypes of virus
  • Sporadic cases and small outbreaks will continue to occur depending on the distribution of susceptible individuals
• Rapid confirmation of vaccine reactions (through molecular techniques) is important in elimination settings in cases where there was vaccination in response to a recent exposure or potential exposure

Transmission patterns
• Endemic: Multiple transmission pathways lead to multiple lineages within endemic genotype(s)
• Elimination: Few cases and multiple imported genotypes with no endemic genotype
• Reintroduction: Rapid spread of genetically homogenous viruses
What Virologic Surveillance Data Tell Us About Measles Transmission Patterns

- Endemic transmission of measles: Multiple transmission pathways lead to multiple lineages within endemic genotype(s)
  - Example: China

- Elimination of measles: Few cases and multiple imported genotypes with no endemic genotype
  - Examples: Western Hemisphere, Australia, New Zealand, Hong Kong

- Reintroduction following interruption: Rapid spread of genetically homogenous viruses
  - Example: Burkina Faso, Philippines

Limitations of Virologic Surveillance

- Molecular studies can confirm independent sources of infection if different genotypes or clearly distinct lineages are detected.

- Molecular studies alone cannot differentiate between continuous circulation of virus and multiple introductions from the same source.

- Therefore, it is extremely important to conduct high quality case investigations and to classify cases after all of the epidemiologic and laboratory data have been reviewed.

Global Status of Laboratory Support Virologic Surveillance for Wild-type Measles Viruses

- All regions and most countries have conducted baseline virologic surveillance for measles
- Most Regional Reference Labs in the WHO LabNet are capable of PCR and sequence analysis
- Most LabNet labs use Vero/hSLAM for isolation
- PCR and viral isolation are included in all WHO intercountry training courses
- Timely reporting of sequence information (within 2 months) is an accreditation requirement in all regions
- Two global databases have been established
  - WHO (genotypes only)
  - HPA-MeaNS (approaching 8000 sequences) enable almost real-time analysis

Requirements for Virologic Surveillance-1

- Testing should be performed in an accredited laboratory
- In a pre or post elimination settings, the goal is to obtain genetic information from every chain of transmission
  - This can be difficult with sporadic cases, usually because of failure to collect adequate samples in a timely manner
  - Typically, there is greater success with obtaining adequate samples from outbreaks
- Specimens must be taken at first contact with suspected case
- Good specimens for virus detection or virus isolation: throat swab, nasal wash or aspirate, oral fluid, white blood cells (not recommended for safety reasons)
- Other samples in which viral RNA can be detected at lower frequencies: dried blood spots, IgM positive serum

Requirements for Virologic Surveillance-2

- An important pre-requisite is to conduct baseline virologic surveillance in all countries to document endemic genotype(s) that are present before the accelerated control measures required for elimination are initiated
- Testing archival samples may be useful if these specimens are available

Standard Methods for Measles Molecular Epidemiology of Measles

<table>
<thead>
<tr>
<th>N</th>
<th>P/C/V</th>
<th>M</th>
<th>F</th>
<th>H</th>
<th>L</th>
</tr>
</thead>
</table>

RT-PCR and Sequence targets:

- N and H genes are the most variable regions on the measles genome.
  - Amplify and sequence the 450 nt. Coding for the COOH terminal 150 amino acids of the N protein from all specimens (Minimum amount of sequence needed for reporting genotype).
  - Amplify and sequence the entire coding region of the H gene from selected isolates
### Measles Cases with Genotype D8 USA, 2010

<table>
<thead>
<tr>
<th>Week</th>
<th>State</th>
<th>Accession</th>
<th>Source</th>
<th>Country</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linn.</td>
<td>JN712282</td>
<td>MVi</td>
<td>California</td>
<td>D8</td>
</tr>
<tr>
<td>2</td>
<td>Linn.</td>
<td>JN712282</td>
<td>MVi</td>
<td>California</td>
<td>D8</td>
</tr>
<tr>
<td>3</td>
<td>Linn.</td>
<td>JN712282</td>
<td>MVi</td>
<td>California</td>
<td>D8</td>
</tr>
</tbody>
</table>

此外，还有其他一些与Measles Cases相关的条目，详细信息请参阅原始文档。

### Measles Cases with Genotypes USA: 2009-2010 (YTD)

<table>
<thead>
<tr>
<th>Week</th>
<th>State</th>
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<th>Source</th>
<th>Country</th>
<th>Genotype</th>
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<td>Linn.</td>
<td>JN712282</td>
<td>MVi</td>
<td>California</td>
<td>D8</td>
</tr>
</tbody>
</table>

另请参阅原始文档获取更多详情。

### WHO Measles Reference Sequences and Accession Numbers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain</th>
<th>Type</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>B</td>
<td>B-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>C</td>
<td>C-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>D</td>
<td>D-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>E</td>
<td>E-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>F</td>
<td>F-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>G</td>
<td>G-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
<tr>
<td>H</td>
<td>H-1</td>
<td>Virus</td>
<td>J01987</td>
</tr>
</tbody>
</table>

### Virologic Surveillance for Measles, USA: 2002-2010 (9/1/10)

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of cases</th>
<th>Number of outbreaks</th>
<th>Number of genotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>44</td>
<td>8 (18%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>2003</td>
<td>56</td>
<td>7 (24%)</td>
<td>3 (42%)</td>
</tr>
<tr>
<td>2004</td>
<td>37</td>
<td>6 (26%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>2005</td>
<td>66</td>
<td>12 (48%)</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>2006</td>
<td>55</td>
<td>14 (45%)</td>
<td>4 (28%)</td>
</tr>
<tr>
<td>2007</td>
<td>43</td>
<td>7 (26%)</td>
<td>4 (30%)</td>
</tr>
</tbody>
</table>

这些数据来自CDC的Virologic Surveillance for Measles, USA: 2002-2010 (9/1/10)。
Measles Cases with Genotype D9 USA, 2010

- California 2/16/2010
- Nebraska 5/17/2010
- Hong Kong.CHN/07.10 from PHL
- California 5/12/2010 (CA MV-2897)
- Region11-PHL
- VIC.AUS 99 D9

Thanks to colleagues at PHL, Hong Kong, RITM, Manila, and WPRO

Measles Cases with Genotype H1 USA, 2010

- California 5/12/2010
- Region11-PHL
- VIC.AUS 99 D9

Thanks to colleagues at Health Canada

Measles Genotypes in Latin America: 2010

- 2010: D4, Brazil (Para) from ARG
- 2010: D3, Brazil from ARG
- 2010: B3, Argentina from SA

Thanks to colleagues at Fiocruz and Inst. Carlos Malbran

Use of conventional and real time PCR for case classification and molecular surveillance

Testing Scheme for Confirmation of Measles Infection

- Throat or Nasal Swab, Ulime
- Virus Isolation
- RNA extraction, RT-PCR
- Measles and Rubella IgM
- Sequence Analysis for Genotype
- Measles IgG
- Avidity
- Vaccine or Wildtype

Utility of New Assays in the Measles Diagnostics Tool Box

<table>
<thead>
<tr>
<th>Classification</th>
<th>Clinical</th>
<th>IgM</th>
<th>IgG</th>
<th>Avidity*</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine reaction</td>
<td>Fever/Rash</td>
<td>P</td>
<td>P or N</td>
<td>Low</td>
<td>Positive and Sequence indicates vaccine strain</td>
</tr>
<tr>
<td>Vaccine reaction</td>
<td>Fever/Rash</td>
<td>N</td>
<td>P</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Measles</td>
<td>Meets CCD*</td>
<td>P</td>
<td>P or N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measles (but recently vaccinated)</td>
<td>Modified*</td>
<td>P</td>
<td>P</td>
<td>Low can confirm IgM</td>
<td>Positive PCR can confirm IgM</td>
</tr>
<tr>
<td>Measles-Primary Vaccine Failure</td>
<td>Meets CCD*</td>
<td>P</td>
<td>P or N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary vaccine failure</td>
<td>Modified*</td>
<td>P or N</td>
<td>P</td>
<td>High</td>
<td>Positive PCR can confirm case</td>
</tr>
</tbody>
</table>

* clinical case definition (CCD)
* modified or mild presentation does not meet CCD
* Collection of sample ≤ 3 weeks after rash onset
PCR

The MeV 214-216 forward and the reverse primer pair was the most sensitive set for detecting measles RNA and detected all genotypes tested at 10^4 copies of viral RNA.

Results and Conclusions

- The primer sets were tested against RNA from 11 different genotypes of measles. RNA concentrations were determined by real time RT-PCR.

- The MV 60 and MV 63.3 primer pair amplified all of the virus genotypes tested at 10^4 copies.

- The MeV 214-216 forward and the reverse primer pair was the most sensitive set for detecting measles RNA and detected all genotypes tested at 10^4 copies of viral RNA.

New primers amplify 10^4 copies of RNA template

- RT-PCR was performed with MeV216 and MeV214

- Lane 1: MW ladder
- Lane 2: Pos control
- Lane 3: Pos sample
- Lane 4: Negative (water) control

Version 2 of the Measles Genotyping Kit: Results

Version 2 of the Measles Genotyping Kit: Control RNA

Location of primer binding sites relative to sequencing window

Improving Primers for Measles Genotyping PCR

Location of primer binding sites relative to sequencing window

Testing Scheme for Measles RT-PCR
Since the previous RNA control did not contain the entire 3' non-coding region of the MeV mRNA, a new positive control RNA was synthesized to be used with MeV 214-216.

**Version 2.0 of the Measles Genotyping kit:**

- In this version, the forward and reverse primers have been modified to increase sensitivity and reduce background and the synthetic positive control RNA has been modified. The reaction conditions, primer and template concentrations, and cycling parameters are the same as those used with the previous version of the kit.
- This new primer set will not amplify the synthetic RNA control supplied in the previous version of the kit. Amplification from infected cell RNA will not be affected.
- Measles RNA for RT-PCR can be extracted from either infected cells or clinical samples.
- Primers MeV214 and MeV216 are designed to amplify a 634 nucleotide region coding for the 3' terminus of the nucleoprotein (N) gene in a standard RT-PCR reaction. The positive control RNA, MeV-N3in included in this kit, is a synthetic RNA transcript of the N gene of measles and it is provided to serve as a positive internal control in the RT-PCR reactions. The control RNA has been modified so that the RT-PCR reaction will produce a larger PCR product (854 nucleotides) than the PCR product produced from measles RNA, thereby providing a means to identify laboratory contamination of the RT-PCR reaction.

**Kit Contents:**
- Forward primer: MeV216, 25ul Stock Solution 200uM
- Reverse primer: MeV214, 25ul Stock Solution 200uM
- Dried Positive Control RNA, MeV-N3in, 10^11 copies
- 1ml of 1X nuclease-free TE

**The sequences of the primers are:**
Forward primer: MeV 216, 5'-TGG AGC TAT GCC ATG GGA GT-3'
Reverse primer: MeV 214, 5'-TAA CAA TGA TGG AGG GTA GG -3'

**To make working stocks of primers MeV214 and MeV216 for RT-PCR:**
- Add 90ul nuclease-free water to 10ul of MeV214
- Add 90ul nuclease-free water to 10ul of MeV216
- Concentration = 20uM
- Store at -20ºC
- Use 0.5 – 1ul per RT-PCR reaction as per RT-PCR protocol.

**Rehydration of measles positive control RNA, MeV-N3in**

To make master stock:
- Add 100ul nuclease-free TE and vortex tube.
- Place at 55ºC for 10 minutes and vortex, again.
- Make 10ul aliquots.
- Concentration = 10^8 copies/ul
- Store at -70ºC

To make working stock:
- Add 90ul nuclease-free TE to the 10ul master stock.
- Concentration = 10^7 copies/ul
- Store at -20ºC for short-term and at -70ºC for long-term.
- Use 1ul per RT-PCR reaction.

**Background: TaqMan Real time RT-PCR**
- Probe contains 5'fluorophore and 3' quencher
- Forward and reverse primers are not modified and are the same as those used in standard RT-PCR.
- Relatively short amplicons (typically less than 100nt)
- Probe is degraded by 5' to 3' exonuclease activity of the Taq polymerase
- Consumption of probe results in fluorescence
- Fluorescence is measure at each cycle (typically 40)
- Threshold cycle (Ct) is cycle at which fluorescence crosses the threshold.

**Detection of Measles RNA by Real Time RT-PCR**
- Real-time assays can detect 10-100 copies of viral mRNA/sample in a high throughput format, results in 3-4 hours
- Can help to confirm a case when serologic results are inconclusive
- Negative results do not rule out a case
- Standard RT-PCR is less sensitive than real time RT-PCR.
- Sequence information from the standard PCR product required for genotype and confirmation of vaccine reactions.
Primers and Probes for Measles Real time RT-PCR and for Reference Gene Control

**MeV N Gene**

Make a 15 µM stock of each forward and reverse primer; final concentration is 300 nM.

- Forward Primer (MVN1139F): 5’ TGG CAT CTG AAC TCG GTA TCA C 3’
- Reverse Primer (MVN1213R): 5’ TGT CCT CAG TAG TAT GCA TTG CAA 3’
- Probe (MVNP1163P): 5’ FAM CCG AGG ATG CAA GGC TTG TTT CAG A BHQ 3’

**Human RNase P Gene (Reference gene)**

Make a 15 µM stock of each forward and reverse primer; final concentration is 300 nM.

- Forward Primer (HURNASE-P-F): 5’ AGA TTT GGA CCT GCG AGC G 3’
- Reverse Primer (HURNASE-P-R): 5’ GAG CGG CTG TCT CCA CAA GT 3’
- Probe (BHQ1HURNASE-P): 5’ FAM TTC TGA CCT GAA GGC TCT GCG CG BHQ 3’

Measles Real time RT-PCR on ABI 7500

**Measles Standards**

![Amplification Plot](image1)

**RNaseP Standards**

![Amplification Plot](image2)

Measles Real time RT-PCR: Interpretation

<table>
<thead>
<tr>
<th>Viral gene (Ct)</th>
<th>RNase P (Ct)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct &lt; 40</td>
<td>Ct &lt; 40</td>
<td>Positive</td>
</tr>
<tr>
<td>Ct &lt; 40</td>
<td>Undetermined</td>
<td>Positive</td>
</tr>
<tr>
<td>Undetermined</td>
<td>Ct &lt; 40</td>
<td>Negative</td>
</tr>
<tr>
<td>Undetermined</td>
<td>Undetermined</td>
<td>Indeterminate</td>
</tr>
</tbody>
</table>

-Ct is average Ct from 2 or 3 replicates/sample
-Positive if 2/3 replicates are <40
-If testing in duplicate, repeat if one Ct is <40 and the other is Und.
-Repeat Indeterminate samples with fresh RNA extraction if possible

Measles Real time RT-PCR on ABI 7500: Standard Curve

![Amplification Plot](image3)

Samples from Suspected Measles Cases, USA, 2009-2010 (through Sept 10)

- Samples for real time RT-PCR: 266 (US only)
  - 79 positive (29%)
  - 6 indeterminate
  - 180 negative
- Virus isolation attempts: 271 (includes international)
  - 2009: 36 positive from 210 samples (17%)
  - 2010: 7 positive from 61 samples (11%)

Shipping viral isolates on filter paper

Drying cells infected with measles or rubella viruses onto specimen paper

**Purpose:** To submit measles or rubella isolates to the Regional or Global Specialized Laboratory for sequence analysis and genotyping. Once the samples are dry, the samples can be stored at 4°C. Samples can be shipped at room temperature.

May also be used to transport proficiency panels for RT-PCR assays

**Important:** Both measles and rubella viruses have been recovered from specimen paper. Treat samples as infectious material.

CDC is testing inactivation protocols as well as the FTA cards that are similar to those used by the polio labs.
What about infectivity?
A non-infectious sample would be useful for a number of purposes including shipment of PT panels and control RNA for RT-PCR.

Whatman FTA elute micro card
- Chemically treated to lyse cells and denature proteins on contact.
- Nucleic acids are protected from microbial and fungal attack.
- Samples are not infectious.
- Samples can be stored and shipped at room temperature.
- Extract RNA using Qiagen Viral RNA Mini kit.

Measles RNA is stable for at least one month on FTA cards
- Measles RNA is stable for at least one month on FTA cards.
- PCR products are stable for at least 1 month.
- PCR reactions were incubated at RT, 4C and 37 C for up to 1 month with no degradation of PCR product.
- PCR reactions can be shipped to sequencing lab at 4C or RT.
- Place thin-walled tube in sturdy container (e.g. 1.5 ml Eppendorf).

Thank You
- Measles, Mumps, Rubella and Herpesviruses Laboratory Branch, DVD/CDC
  - William Bellini, Jennifer Rota, Yuan Tian, Luis Lowe, Elena Lopareva, Bethina Bankamp, Lauren, Rebecca, McNail Byrd, Joe Icenogle, Emily Abernathy, Lijuan Hao
- Epidemiology Branch, DVD/CDC
- Kathy Gallagher, Susan Redd, Al Barnes
- Global Immunization Division/CDC
- National Microbiology Laboratory, Canada
- PAHO LabNet: Carlos Castillo, Cristina Marsigli
- FiCruz: Marinda Siqueira
- Inst. Carlos Malbran: Elsa Baumeister
- WHO/HQ, RRL and NLS
- State and Local Public Health Labs, USA