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SURVEILLANCE ET CONTRÔLE DE LA ROUGEOLE: CARACTÉRISATION DU VIRUS ET DE L'IMMUNITÉ HUMORALE

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À ma femme

À mes parents

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TABLEAU DES ABRÉVIATIONS

A – adénine

ADN – acide désoxyribonucléique

ARN – acide ribonucléique

ARNm – acide ribonucléique messager

bp – *base pairs*

C – cytosine

DNA – *deoxyribonucleic acid*

ELISA – *Enzyme-linked Immunosorbent Assay*

G – guanine

IgA – immunoglobuline A

IgG – immunoglobuline G

IgM – immunoglobuline M

IL – interleukine

OD – *optical density*

PCR – *polymerase chain reaction*

PBL – *peripheral blood leukocytes*

RNA – *ribonucleic acid*

RT-PCR – *reverse transcription polymerase chain reaction*

SLAM – *signaling lymphocyte activation molecule*

T – thymidine

UI – unités internationales

INTRODUCTION

Depuis l'introduction de la vaccination l'incidence de la rougeole ainsi que la mortalité due à cette maladie ont continuellement diminués. Plusieurs plans stratégiques ainsi qu'un réseau global de laboratoires spécialisés ont été élaborés par l'Organisation Mondiale de la Santé (OMS) pour renforcer la surveillance ainsi que le contrôle de la rougeole. Ainsi la circulation du virus de la rougeole a pu être interrompue au moins temporairement sur tout le continent américain ainsi que dans de nombreux pays européens et en Australie. Malgré l'énorme progrès vers le contrôle global de la maladie, la rougeole est toujours endémique dans la plus grande partie de l'Afrique et de l'Asie du Sud-Est ainsi que dans différentes régions d'Europe. Par conséquent le virus pourrait être réintroduit dans des populations insuffisamment protogées, même après élimination locale du virus.

Il a été démontré auparavant que l'immunité conférée par infection naturelle est plus robuste qu'après vaccination. Dans ce contexte nous avons analysé l'immunité humorale contre la rougeole induite par vaccination chez des adolescents du Luxembourg, afin d'évaluer si le déclin des taux d'anticorps après vaccination pourrait compliquer le contrôle de la maladie dans l'avenir. Les taux d'anticorps ont été mesurés dans deux sérums prélevés auprès des mêmes individus dans un intervalle de 6.8 ans. D'un autre côté nous avons aussi analysé l'effet d'une réexposition à des souches sauvages chez des convalescents vivant en proche contact avec des enfants qui ont contracté la rougeole. Pour cela les taux d'anticorps ont été comparés dans des sérums prélevés avant et après le contact avec un patient. Aussi nous avons évalué un test ELISA développé pour détecter les IgG spécifiques la rougeole dans du fluide buccal, qui pourrait être utile pour mesurer l'immunité contre la rougeole sans prélèvement de sang.

L'épidémiologie moléculaire s'avère être un outil majeur pour identifier les chaînes de transmission ainsi que les sources d'importations du virus de la rougeole. De plus l'analyse génétique des virus permet de vérifier si la circulation des souches endémiques a pu être interrompue par une campagne de vaccination. Afin de faciliter le génotypage sur un grand nombre d'échantillons ainsi qu'à palier à l'absence de facilités de séquençage, nous

avons développé une méthode simplifiée pour faire la distinction entre les clades et génotypes du virus de la rougeole considérés actifs (A, B3.1.-B3.2., C2, D2-D9, G2-G3, et H1-H2). Cette méthode est basée sur 6 PCR multiplex différentes, qui produisent des fragments PCR avec une longueur caractéristique des différents clades ou génotypes. La méthode a été développée en utilisant des isolats viraux appartenant aux différents génotypes et évaluée sur des échantillons cliniques prélevés auprès de patients de la rougeole.

SYNTHÈSE BIBLIOGRAPHIQUE

I. Le virus de la rougeole

I.1. Taxonomie

Le virus de la rougeole est un virus enveloppé à ARN simple brin de polarité négative. Il appartient au genre des morbillivirus, et à la famille des *paramyxoviridae*, subdivisée en deux sous-familles, les *paramyxovirinae* et les *pneumovirinae* (Figure 1A). Ces derniers se distinguent par la taille de leurs nucléocapsides et par leur structure génomique.

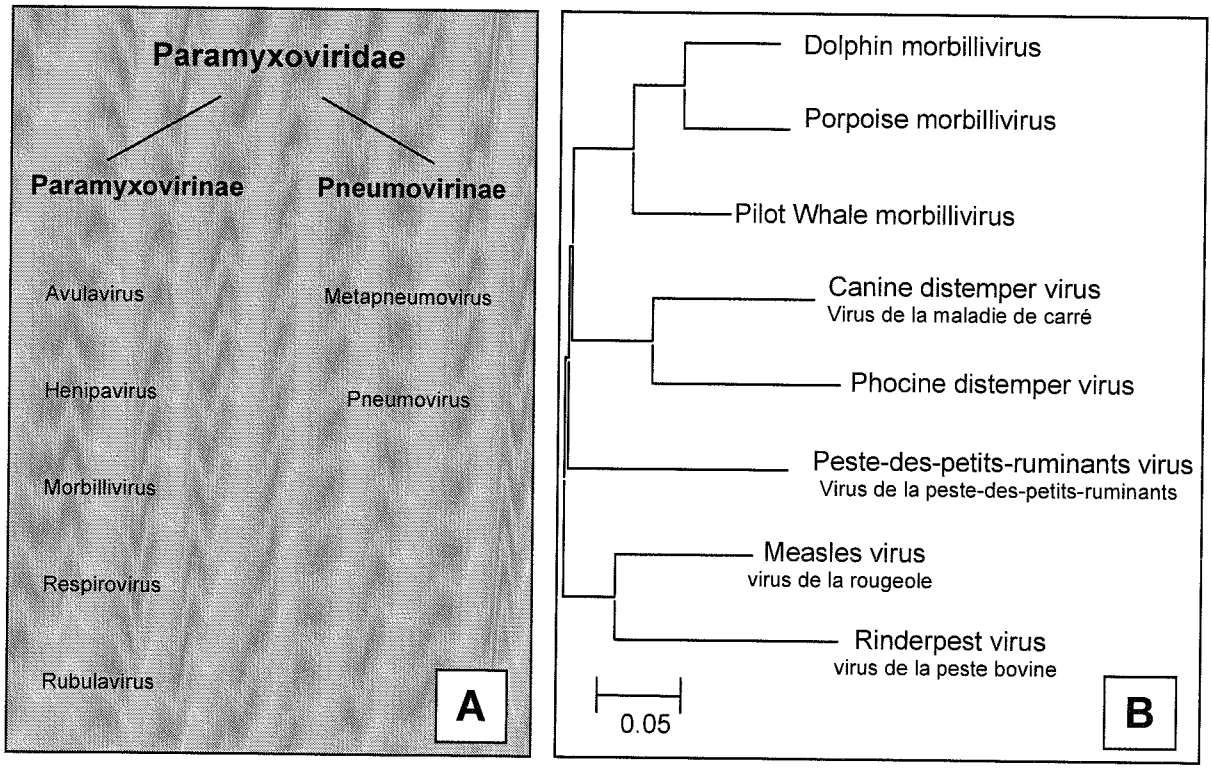

Figure 1 : (A) Subdivision des *Paramyxoviridae* en deux sous-familles regroupant différents genres. (B) Distance génétique entre différents morbillivirus sur la base de 230 nucléotides du gène N.

Les morbillivirus appartiennent à la sous-famille des *paramyxovirinae* et regroupent le virus de la rougeole, le virus de la maladie de Carré, le virus de la peste bovine, le virus de la peste-des-petits-ruminants ainsi que différents morbillivirus découverts plus récemment auprès de mammifères maritimes tels

que les dauphins, les baleines et les phoques (Fig. 1B) [De Swart et al. 1995; Kennedy 1998]. Ils se distinguent des autres membres de cette sous-famille par l'absence d'activité neuraminidase.

I.2. Historique

On suppose que la co-habitation étroite entre hommes et bovins est à l'origine du virus de la rougeole. Ces circonstances auraient facilité l'introduction du virus de la peste bovine chez les humains [Norrby et al. 1992]. Cependant l'étude de la propagation du virus de la rougeole dans des populations isolées suggère qu'au minimum 300 000 à 400 000 individus furent nécessaires pour permettre une circulation continue du virus chez l'homme [McNeill 1976]. Des communautés de cette importance seraient créées pour la première fois après le regroupement de populations dans l'Égypte antique ainsi qu'en Sumérie et en Chine. Aujourd'hui l'homme est le seul réservoir naturel du virus de la rougeole.
La première description précise de la rougeole a été faite par Abu Becr, un médecin arabe du IX^{e} siècle, connu sous le nom de Rhazes de Bagdad [Rhazes 1748]. Le premier isolat du virus de la rougeole, la souche Edmonston, a été obtenu en 1954 par Enders et Peebles à partir du sang d'un enfant infecté [Enders et Peebles 1954].

I.3. Structure et Génome

Le virus de la rougeole est un virus enveloppé, généralement sphérique ou pléomorphique d'environ 100-300 nm de diamètre. Le génome du virus est constitué d'un seul brin d'ARN de polarité négative, comprenant 6 gènes codant pour 9 protéines: la nucléoprotéine (N), l'hémagglutinine (H), la protéine de fusion (F), la protéine de matrice (M), la protéine large (L), la phosphoprotéine (P), ainsi que les trois protéines non structurales R, C et V [Griffin 2001]. L'ARN viral est encapsidé dans un complexe de nucléoprotéines, le nucléocapside auquel sont aussi associées les protéines L et P (Fig. 2). Par l'intermédiaire des

protéines de matrice ces ribonucléoprotéines sont attachées à une couche bilipidique, portant les protéines H et F.

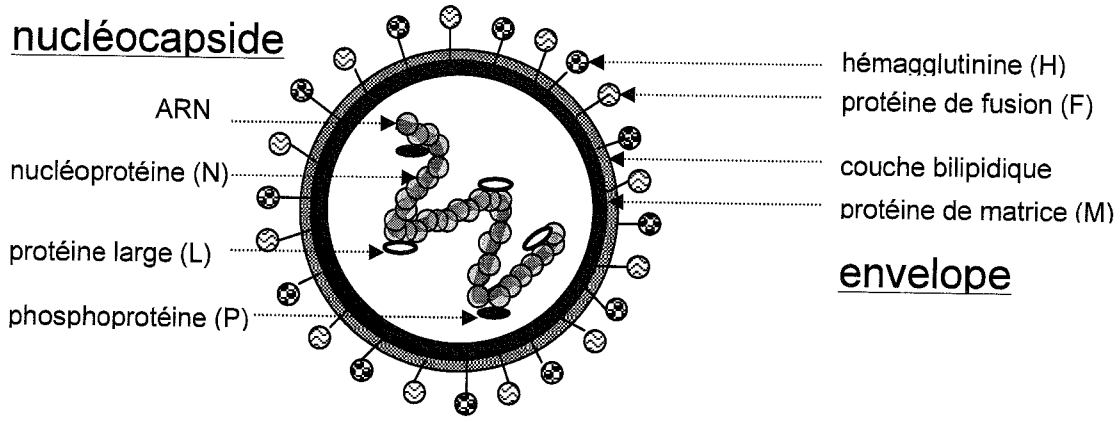

Figure 2 : Structure schématique du virus de la rougeole.

Le génome non segmenté de 15894 ribonucléotides est précédé par une séquence non codante de 55 nucléotides (leader sequence) et se termine par une suite de 36 nucléotides non transcrits (trailer sequence). Les 6 gènes, N, P, M, F, H et L sont séparés par des régions intergéniques contenant de courtes séquences conservées [Griffin 2001] (Fig. 3).

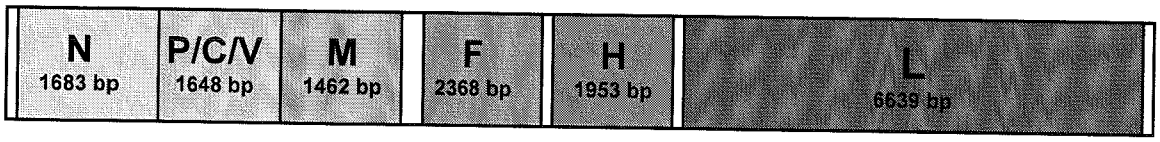

Figure 3 : Structure génomique du virus de la rougeole.

I.4. Cycle infectieux

L'infection cellulaire débute par l'adhésion des protéines H, ancrées dans l'enveloppe bilipidique du virus, aux récepteurs cellulaires CD46 ou SLAM [Dorig et al. 1993; Tatsuo et al. 2000]. En général les souches sauvages peuvent seulement se lier au récepteur SLAM (CD150w), tandis que les souches

vaccinales peuvent également entrer dans les cellules à travers la protéine membranaire CD46 [Yanagi 2001]. Différentes études suggèrent qu'il existe d'autres récepteurs cellulaires du virus de la rougeole non identifiés à ce jour [Yanagi 2001; Waku Kouomou et Wild 2002]. La fusion des membranes virales et cellulaires, par l'intermédiaire des protéines F et H, résulte en l'introduction des particules ribonucléoprotéiques du virus dans le cytoplasme de la cellule hôte [Wild et al. 1991]. A l'intérieur des cellules, l'ARN viral (ARNv) reste englobé de nucléoprotéines pendant la transcription ainsi que la réplication du génome [Ray et Fujinami 1987]. La transcription est toujours initiée à l'extrémité 3' de l'ARNv par la polymérase virale, composée de la sous-unité catalytique (protéine L) et du co-facteur, la protéine P. L'expression génétique est régulée par la présence de signaux d'arrêt et de reprise aux extrémités de tous les gènes viraux [Whelan et al. 2004]. Puisque les signaux d'arrêt peuvent provoquer le détachement de la polymérase, la quantité des différents ARNm produits dépend de la distance par rapport au seul point d'initiation à l'extrémité 3' [Cattaneo et al. 1987; Schneider-Schaulies et al. 1989]. La phase de réplication commence après la traduction des premiers transcrits d'ARNm, et l'accumulation des protéines virales dans le cytoplasme. Pour la réplication du génome complet du virus, la polymérase virale ignore les signaux d'arrêt à la fin de la séquence leader et des différents gènes. C'est surtout la présence de nucléoprotéines qui semble favoriser la réplication [Banerjee 1987]. Les protéines C et V sont aussi impliquées dans la régulation de la transcription et la réplication [Liston et al. 1995].

Durant la réplication, des protéines N s'associent à l'ARNv produit pour former des particules ribonucléoprotéiques, auxquelles se lie par après le complexe protéique P-L [Kingston et al. 2004]. Ces complexes sont transportés vers la membrane cellulaire, par l'intermédiaire de la protéine M qui s'associe à des filaments d'actine croissants [Bohn et al. 1986; Hirano et al. 1992]. Les protéines M se lient d'un autre côté aux parties cytoplasmiques des glycoprotéines virales H et F, elles-mêmes attachées à la membrane cellulaire. Ce phénomène induirait le bourgeonnement du virus.

II. La maladie de la rougeole

II.1. Pathologie

Le virus de la rougeole se transmet par des gouttelettes de sécrétions respiratoires. Les premières cellules infectées sont celles de la muqueuse respiratoire [Riley et al. 1978]. Les macrophages ainsi que les cellules dendritiques infectés au niveau de la voie respiratoire vont ensuite transporter le virus dans les ganglions lymphatiques locaux [Sherman et Ruckle 1958]. La réplication du virus dans les ganglions lymphatiques induit une première virémie qui provoque l'infection du système réticulo-endothélial (2-3 jours après l'infection).

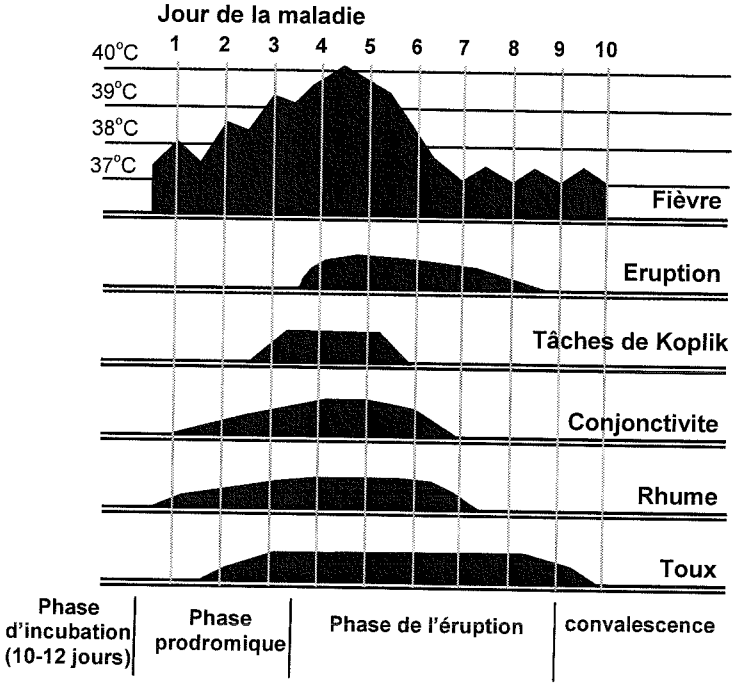

Figure 4 : Signes cliniques de la rougeole typique (d'après [WHO - World Health Organization 2006]).

Les monocytes principalement, mais aussi certains lymphocytes sont infectés à ce stade et contribuent ainsi à la diffusion du virus dans tout l'organisme. Il

s'ensuit au 5-7^{e} jour une virémie secondaire menant à une infection généralisée, avec localisation prédominante au niveau des tractus respiratoire, cutané et conjonctival [Ruckle et Rogers 1957].

L'étape initiale de la maladie, qui correspond à la dissémination du virus dans l'organisme, est silencieuse. La phase prodromique, caractérisée par des symptômes de fièvre, de toux, de conjonctivite et de rhume commence en moyenne 10-12 jours après l'infection (Figure 4). Un ou deux jours avant l'éruption, des tâches de Koplik, signes pathognomoniques de la rougeole, apparaissent sur la muqueuse buccale et persistent un ou deux jours après l'éruption [Koplik 1896]. L'éruption, de type érythémateux maculo-papulaire, apparaît d'abord à la racine des cheveux et descend ensuite progressivement vers le visage, le cou, la partie inférieure du corps, et les extrémités. L'éruption s'efface dans l'ordre d'apparition à partir du troisième jour, laissant des zones brunes décolorées.

La fréquence de complications est relativement élevée, et varie en fonction de l'état de santé général et de l'âge des patients. Les complications les plus fréquentes sont la diarrhée, l'otite ou la pneumonie. Plus rarement les patients peuvent aussi être atteints de différents types d'encéphalites: encéphalite aiguë (en moyenne 6 jours après l'éruption), encéphalite aiguë progressive (environ 6 mois après l'infection), panencéphalite sclérosante subaiguë (en moyenne 7 ans après l'infection). Le taux de mortalité peut atteindre 30% dans certaines régions du monde, plus particulièrement dans les pays en voie de développement [WHO - World Health Organization 1999a]. Dans la majorité des cas mortels les patients meurent de surinfections virales ou bactériennes, facilitées par un état d'immunosuppression suivant la maladie de la rougeole.

II.2. Réponse immunitaire

Le virus sauvage de la rougeole induit une réponse humorale et cellulaire qui protège les convalescents à vie contre une réinfection [Panum 1939; Graves et al. 1984; Sissons et al. 1985]. Il a été montré que l'administration passive de

gammaglobulines spécifiques de la rougeole peut inhiber l'infection par le virus [Janeway 1945], ce qui suggère que l'immunité humorale est suffisante pour protéger les individus contre une réinfection. Cependant, l'évolution progressive de la maladie chez des personnes qui souffrent d'anomalies congénitales ou acquises de la réponse cellulaire, indique que cette dernière est indispensable pour l'élimination du virus après infection [Kaplan et al. 1992].

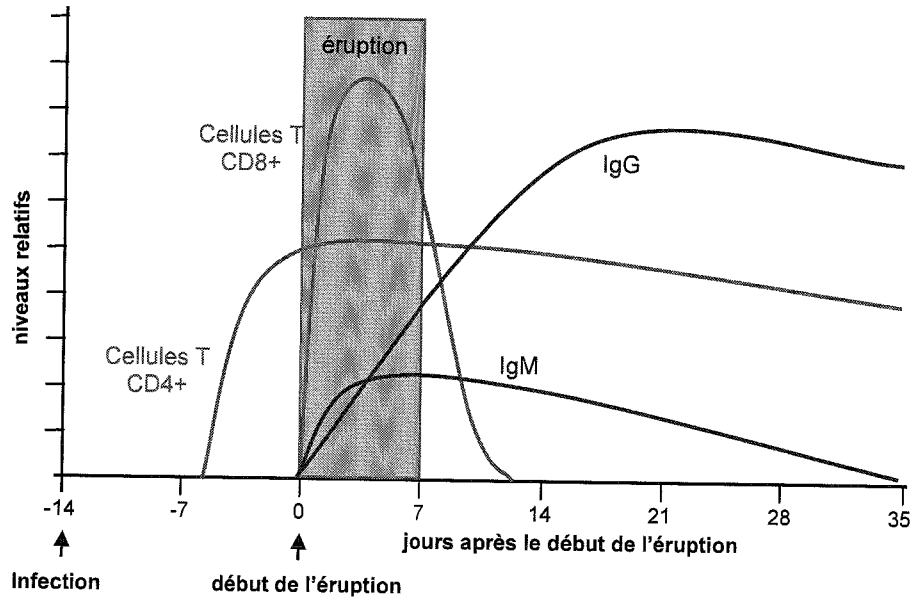

Figure 5 : Réponse immunitaire au cours d'une rougeole typique (d'après [WHO - World Health Organization 2006])

Des anticorps spécifiques du virus de la rougeole sont détectables dés l'apparition de l'éruption [Bech 1959] (Figure 5). Les IgM sont produits en premier et indiquent une primo-infection. Les anticorps IgG et IgA, détectables quelques jours plus tard dans le sérum ainsi que dans les sécrétions, persistent à vie. Les IgM quant à eux disparaissent dans les 8 semaines après l'éruption. Les IgG sont dirigés contre toutes les protéines virales, mais majoritairement contre la nucléoprotéine [Bouche et al. 2002]. Cependant seuls les IgG spécifiques des protéines H et F contribuent à la neutralisation du virus [De Swart et al. 2005].
L'analyse des cytokines dans le sang ainsi que la détection directe des différents types de cellules T prouvent que des cellules T CD8+ ainsi que des cellules T

CD4+ de type I et II sont activées au cours de la maladie. Les cellules CD8+, responsables de l'éruption, seraient activées pendant la phase prodromique. Parmi les cellules T CD4+, celles du type I semblent être dominantes pendant l'éruption, tandis que celles du type II dominent pendant la phase de convalescence [Griffin 1995].

Paradoxalement l'induction d'une réponse immunitaire efficace et de longue durée contre le virus de la rougeole est suivie d'un état d'immunosuppression général qui peut persister pendant plusieurs mois [Tamashiro et al. 1987]. Bien que le mécanisme de cette immunosuppression n'ait pas encore été complètement élucidé, plusieurs voies du système immunitaire semblent être impliquées [Schneider-Schaulies et al. 2001]. Ainsi il a été montré que la rougeole s'accompagne d'une lymphopénie transitoire ainsi que d'un blocage de la prolifération des lymphocytes [Fugier-Vivier et al. 1997; Schnorr et al. 1997]. D'autre part, l'absence de la réaction d'hypersensibilité retardée (anergie à la tuberculine) démontrée par von Pirquet déjà en 1908 [Pirquet 1908], ainsi que la quantité réduite de cytokines impliquées dans l'activation des cellules CD4+ de type I dans le sérum [Griffin et Ward 1993] indiquent que la réponse cellulaire du système immunitaire est affectée après convalescence. Ces phénomènes ont partiellement pu être expliqués par l'apoptose des lymphocytes et cellules dendritiques infectés, ainsi que par une inhibition de la différenciation et par une production réduite d'IL-12 dans des cellules en contact avec le virus ou d'autres cellules infectées [Karp et al. 1996; Atabani et al. 2001].

II.3. Diagnostic

Cliniquement un cas de rougeole est défini de la manière suivante: éruption généralisée d'une durée supérieure à 3 jours, accompagnée de fièvre supérieure à 38,5°C et d'une toux, d'un coryza ou d'une conjonctivite [WHO - World Health Organization 2006]. La similitude des symptômes par rapport à d'autres maladies (par exemple la rubéole) nécessite pourtant un test diagnostic dans un laboratoire.

Dans la plupart des cas les anticorps IgM spécifiques de la rougeole sont mesurés à l'aide d'un test ELISA pour confirmer le diagnostic clinique. En général les anticorps IgM sont détectables dés le début de l'éruption (Figure 5). En moyenne, ils atteignent un maximum 7-10 jours après le début de l'éruption et restent détectables pendant 8 semaines tout au plus. Un sérum prélevé dans les trois premiers jours qui suivent l'éruption peut pourtant donner un résultat faussement négatif, surtout si la sensibilité du test ELISA est réduite. Une augmentation d'IgG dans deux sérums consécutifs - dont le premier fut prélevé dans les 2 semaines qui suivant l'éruption - peut aussi indiquer/confirmer une infection par le virus. Alors que les anticorps sont généralement mesurés dans le sérum, la détection d'IgM et IgG dans la salive a été évaluée comme alternative [Nokes et al. 2001; Kremer et Muller 2005].

Les cas de rougeole peuvent aussi être confirmés par RT-PCR. Dans ces cas l'ARN viral peut être isolé à partir de différents échantillons cliniques. La sensibilité de cette technique dépend cependant du type d'échantillon, du temps écoulé depuis le début de l'éruption et du traitement subi par l'échantillon [van Binnendijk et al. 2003]. En général les échantillons prélevés au niveau des muqueuses nasopharyngales contiennent la plus grande quantité d'ARN viral. Alternativement l'ARN peut aussi être isolé à partir d'urine, de sérum, de plasma sanguin, des leucocytes du sang périphérique ou de sang séché sur papier filtre [El Mubarak et al. 2004]. Dans tous les cas l'échantillon doit être prélevé au plus tard dans les 7 jours qui suivent l'éruption. De plus, les échantillons doivent être conservés à des températures minimales (optimalement à –80°C), tout en évitant plusieurs cycles de congélation/décongélation avant l'extraction d'ARN.

La fraction cellulaire de l'urine ainsi que les leucocytes du sang périphérique et les échantillons nasopharyngaux peuvent aussi être utilisés pour l'isolement du virus en culture cellulaire. Les cellules Vero (cellules épithéliales de rein de singe) ont été longtemps utilisées pour l'isolation du virus de la rougeole. En 1993 Kobune et collègues ont montré que les cellules B95a (lignée cellulaire B de singe) étaient maintes fois plus sensibles que les Vero pour l'isolation virale [Kobune et al. 1990]. Aujourd'hui on sait que ceci était dû à la présence du

récepteur des souches sauvages SLAM seulement sur les cellules B95a. Pourtant l'infection permanente de cette lignée cellulaire par le virus Epstein-Barr a toujours été considérée comme un désavantage. Voilà pourquoi l'OMS recommande aujourd'hui l'utilisation de cellules Vero, transfectées avec le récepteur SLAM (Vero SLAM) pour l'isolation du virus de la rougeole [WHO - World Health Organization 2005].

II.4. Vaccination

II.4.1. Vaccins à virus vivants atténués

Le premier vaccin contre la rougeole a été produit par le groupe d'Enders à la fin des années 1950 par de multiples passages consécutifs de la souche Edmonston sur des cultures primaires de cellules rénales et amniotiques humaines, suivis de plusieurs passages sur embryon de poulet avant l'adaptation du virus aux fibroblastes de poulet [Bellini et al. 1994]. Cette première souche vivante atténuée du virus de la rougeole (Edmonston B) fut brevetée comme vaccin en 1963. Par la suite plusieurs autres souches atténuées furent crées à partir de la souche Edmonston (Schwarz, Moraten, AIK-C, Zagreb) ou d'autres souches sauvages (Changchun-47, Leningrad-16, Shanghai-191, Cam-70) [Bellini et al. 1994]. La majorité d'entre-elles continue à être utilisée comme vaccin vivant atténué contre la rougeole. Aujourd'hui les souches vaccinales contre la rougeole sont souvent combinées à une souche vaccinale contre la rubéole et/ou les oreillons (vaccins ROR).

II.4.2. Autres vaccins contre la rougeole

Bien que les vaccins vivants atténués soient en général très immunogènes et induisent une immunité de longue durée après une seule dose [Krugman 1983], ils engendrent plusieurs désavantages : instabilité biochimique et génétique, neutralisation par des anticorps maternels, ainsi que leurs effets chez des

personnes immunosupprimées [Stittelaar et al. 2002]. Voilà pourquoi plusieurs vaccins alternatifs ont été évalués depuis les premières vaccinations contre la rougeole.

Au début des années 1960 un vaccin inactivé fut préparé à partir de la souche Edmonston par traitement au formol. Cependant l'immunité induite par ce vaccin fut de courte durée et un grand pourcentage de ceux qui furent infectés après la vaccination ont développé une forme sévère de la rougeole, appelée rougeole atypique [CDC - Centers for Disease Control and Prevention 1967]. Ceci mit fin à l'utilisation de ce vaccin.

Des vaccins vivants atténués à hauts titres ont permis de déclencher une réponse immunitaire en présence d'anticorps maternels. Cependant une mortalité infantile plus élevée chez les enfants qui ont reçu ce vaccin ne permit pas non plus de continuer sur cette piste [Holt et al. 1993].

L'administration de souches vivantes atténuées par aérosol semble la manière la plus prometteuse pour résoudre plusieurs problèmes du vaccin classique en ce moment [Dilraj et al. 2000]. Tout d'abord la mise en aérosol directe de virus lyophilisés réduit considérablement sa thermolabilité. De plus cette route d'administration semble moins affectée par la présence d'anticorps maternels [Belyakov et al. 1999]. Finalement il est important de noter que l'utilisation de seringues avec tous les désavantages liés à la contamination et aux déchets ne serait ainsi plus nécessaire.

D'autres approches pour développer des nouveaux vaccins contre la rougeole comprennent les vecteurs viraux, les ISCOM (Immune-stimulating-complex), les vaccins à peptides et les vaccins à ADN [Stittelaar et al. 2002].

III. Surveillance et contrôle de la maladie de la rougeole

III.1. Historique et rôle de l'OMS

D'après les estimations de l'OMS quelques 80 millions de cas de rougeole et plus de 5 millions de décès par an seraient survenus pendant la période ayant précédé la vaccination [WHO/UNICEF 2001]. Depuis l'administration des premiers vaccins contre la rougeole dans les années 1960, la couverture de vaccination globale a progressivement augmenté et par conséquent la morbidité ainsi que le taux de mortalité ont été considérablement réduits [CDC - Centers for Disease Control and Prevention 2006]. Encouragée par l'efficacité de la vaccination, l'OMS a développé avec ses partenaires plusieurs plans de réduction de mortalité et d'élimination régionale de la rougeole. Plus récemment un réseau global de laboratoires travaillant sur la rougeole a été mis en place pour améliorer la surveillance et pour faciliter le contrôle de la maladie au niveau mondial [Featherstone et al. 2003].

III.1.1. Plan d'action pour la lutte contre la rougeole (1990)

En 1990 un plan d'action pour la lutte contre la rougeole a été mis en place par l'OMS ainsi que par les dirigeants des états membres et d'autres organisations mondiales [WHO - World Health Organization 1992]. Ce plan prévoyait une réduction des décès dus à la rougeole de 95% par comparaison avec la période ayant précédé la vaccination ainsi qu'une réduction de 90% des cas de rougeole recensés avant 1995. Bien que cet objectif n'ait pas été atteint au niveau mondial, des efforts considérables surtout dans les Régions des Amériques, d'Europe et du Pacifique occidental ont permis de réduire la morbidité ainsi que la mortalité globales de plus de 63% et 83% en 1998 par rapport à la période ayant précédé la vaccination [WHO - World Health Organization 1999b].

III.1.2. Plan stratégique pour la réduction de la mortalité et l'élimination régionale de la rougeole (2001)

En 2001 un nouveau plan stratégique a été mis en place pour accélérer l'élimination régionale de la rougeole et la réduction de la mortalité [WHO/UNICEF 2001]. Ce plan stratégique avait pour but de :

(i) réduire de moitié le nombre de décès dus à la rougeole de 2000 à 2005 ;
(ii) parvenir à interrompre la transmission autochtone de la rougeole dans les vastes zones géographiques qui ont établi des objectifs en matière d'élimination: la région des Amériques avant la fin de l'an 2000; la région européenne avant la fin de l'an 2007 et la région de la méditerranée orientale avant fin 2010;
(iii) examiner les progrès réalisés et évaluer la faisabilité d'une éradication mondiale de la rougeole.

Les stratégies recommandées pour atteindre ces objectifs étaient:

(i) administrer la première dose de vaccin antirougeole aux cohortes successives de nourrissons ;
(ii) faire en sorte que tous les enfants puissent bénéficier d'un rappel vaccinal contre la rougeole
(iii) renforcer la surveillance de la rougeole en intégrant les données épidémiologiques et les résultats de laboratoire
(iv) améliorer la prise en charge de tous les cas de rougeole.

III.1.3. Réseau global de laboratoires travaillant sur la rougeole

Motivé par le succès du réseau global de laboratoires travaillant sur la polio, l'OMS a commencé à développer un réseau global de laboratoires travaillant sur

la rougeole en 2002, tout en profitant partiellement des structures existantes du réseau polio [Featherstone et al. 2003]. Le réseau est organisé sur 4 niveaux avec différentes responsabilités: laboratoire global spécialisé, laboratoire de référence régional, laboratoire de référence national, laboratoire de référence sub-national. La création d'un tel réseau fut motivée par les cinq objectifs suivants :

(i) développement d'un contrôle de qualité et de procédures standardisées pour la confirmation des cas de rougeole en laboratoire
(ii) mise en place de mécanismes de support et de référence pour les laboratoires du réseau
(iii) possibilité d'entraînement pour le personnel des laboratoires du réseau
(iv) mise en place d'une source de matériel de référence et d'expertise pour le développement et le contrôle de qualité de nouveaux tests diagnostiques
(v) mise en place d'une collection d'isolats du virus de la rougeole pour l'épidémiologie moléculaire ainsi que de sérums de référence pour le contrôle de qualité

III.2. Elimination régionale ou/et éradication globale de la rougeole

Le virus de la rougeole peut être considéré comme éradicable pour les raisons suivantes :

(i) l'homme est le seul réservoir naturel du virus ;
(ii) la rougeole a une expression clinique très marquée ;
(iii) l'infection ainsi que la possibilité de transmission, sont de courte durée ;
(iv) il existe un vaccin efficace qui protège contre l'infection par toutes les souches sauvages

Pourtant le virus de la rougeole est le virus transmissible par aérosol le plus infectieux connu. Pour diminuer la circulation du virus sauvage et par conséquent l'incidence de la maladie, le nombre de personnes susceptibles doit être minimisée à l'aide de stratégies de vaccination efficaces.

IV. Contrôle de la maladie de la rougeole par vaccination

IV.1 Anticorps maternels

Il a été estimé que >95% d'une population doit être protégée immunitairement pour éviter une transmission prolongée du virus de la rougeole [Mossong et Muller 2000]. Pour obtenir un tel taux de protection des stratégies de vaccination optimales doivent être appliquées. L'optimisation des programmes de vaccination actuels consiste pour une grande partie en la détermination de l'âge optimal pour la première vaccination, influencé surtout par la présence d'anticorps maternels [Muller 2001].

Le transfert d'anticorps maternels par le placenta pendant les dernières semaines de grossesse confère aux nouveaux-nés une protection temporaire contre la rougeole. Cependant la durée de protection peut varier considérablement en fonction du niveau socio-économique des différentes régions [Caceres et al. 2000]. Dans les pays industrialisés les anticorps maternels subsistent jusqu'à l'âge de 12 mois en moyenne [Cutts 1990]. Par contre une durée de protection inférieure à 6 mois est fréquente dans des populations avec un niveau socio-économique plus bas [Hartter et al. 2000]. D'autres infections plus fréquentes chez les nourrissons, ainsi qu'un mauvais état de santé de la mère peuvent considérablement réduire la durée de protection par anticorps maternels [Caceres et al. 2000].

IV.2. Taux d'anticorps après vaccination

Il a été montré que les taux d'anticorps après vaccination augmentent avec l'âge de vaccination, du moins pendant les deux premières années de vie [Gans et al. 2001]. L'absence d'anticorps maternels ainsi que la maturité du système immunitaire plus avancée chez des enfants plus âgés, augmentent considérablement la probabilité d'induire une bonne réponse immunitaire par

vaccination. Des études comparatives ont aussi indiqué que l'efficacité d'immunisation peut varier selon la souche virale utilisée, et que le taux de séroconversion augmente avec la dose du virus injectée [Cutts et al. 1995], notamment en présence d'anticorps maternels. D'autre part les taux d'anticorps après vaccination semblent être associés aux différents types de molécules HLA de l'hôte [Jacobson et al. 2003]. Par contre l'état de santé et de nutrition de l'hôte aurait en général peu d'influence sur le développement de la réponse immunitaire [Halsey et al. 1985], bien que l'infection par le VIH puisse diminuer considérablement la réponse immunitaire [Moss et al. 1999].

IV.3. Stratégies de vaccination

L'âge de vaccination est influencé principalement par deux facteurs:

(i) la durée de persistance d'anticorps maternels chez l'enfant
(ii) le risque de contact avec un virus sauvage de la rougeole

Dans les pays industrialisés, les anticorps maternels disparaissent en moyenne avant l'âge de 12 mois. De plus la circulation endogène du virus a pu être interrompue dans la plupart de ces pays. Voilà pourquoi la première vaccination se fait en général à l'âge de 12-15 mois dans ces régions. Dans ces circonstances, l'efficacité de vaccination est estimée entre 90% et 95% [Mossong et Muller 2000]. Pour protéger aussi ceux qui n'ont pas répondu à la première vaccination tous les enfants sont en général réimmunisés à l'âge scolaire.
Dans les pays en voie de développement par contre, les enfants sont souvent vaccinés dès l'âge de 6 mois, pour compenser le risque plus élevé d'exposition au virus sauvage et la durée plus courte de protection par anticorps maternels. Cependant, des anticorps maternels toujours présents chez une partie des vaccinés ainsi que l'immaturité du système immunitaire à cet âge réduisent l'efficacité du vaccin à 85% environ. A part ces facteurs biologiques, des problèmes logistiques, budgétaires ou religieux, ainsi que l'instabilité du vaccin

vivant réduisent souvent davantage la couverture de vaccination dans cette partie du monde [Cutts et al. 1990]. Des campagnes de vaccination visant des groupes d'âge plus larges sont organisées plus ou moins régulièrement pour augmenter le taux de protection dans ces populations.

IV.4. Cas d'échecs de vaccination

Des cas de rougeole auprès de personnes vaccinées sont observés avec des fréquences variables dans différentes régions géographiques [Gustafson et al. 1987; Mathias et al. 1989; Whittle et al. 1999]. Dans ces cas là on fait la distinction entre des cas d'échecs primaires et secondaires de vaccination. Les cas d'échecs primaires sont caractérisés par l'absence de séroconversion après vaccination, et sont dus dans la plupart des cas (i) à la neutralisation du vaccin par des anticorps maternels ou (ii) à l'administration ou au stockage inapproprié du vaccin. Par définition, les échecs secondaires de vaccination résultent d'une perte de protection après séroconversion initiale.

IV.5. Durée de protection après vaccination

L'infection naturelle par des souches sauvages induit une protection à vie contre la rougeole [Panum 1939]. Plusieurs enquêtes prospectives ont montré que le vaccin vivant contre la rougeole induit aussi une protection de longue durée chez la grande majorité des personnes vaccinées, même après une seule dose [Panum 1939; Krugman 1983; Guris et al. 1996]. Cependant les taux d'anticorps induits par vaccination sont nettement plus bas qu'après infection naturelle [Damien et al. 1998]. De plus, les taux d'anticorps semblent diminuer avec le temps [Christenson et Bottiger 1994; Davidkin et Valle 1998; Mossong et al. 2000; Lee et al. 2001], ce qui suggère que les vaccinés puissent perdre leur protection plus ou moins rapidement, en fonction des taux d'anticorps induits au départ. En effet plusieurs épidémies de rougeole ont été décrites dans des populations hautement vaccinées [Gustafson et al. 1987; Markowitz et al. 1989;

Mathias et al. 1989]. De plus des cas de rougeole chez des personnes ayant séroconverti après vaccination suggèrent davantage que les taux d'anticorps peuvent atteindre un niveau trop bas pour protéger contre la maladie [Reyes et al. 1987; Hirose et al. 1997]. Il est donc important de déterminer la durée ainsi que le niveau de protection conférés par la vaccination.

IV.5.1. Taux d'anticorps protecteur

En général les taux d'anticorps sont mesurés dans le sérum pour déterminer le niveau d'immunité d'un individu contre la rougeole. Différents tests ont été développés pour déterminer le taux d'anticorps contre le virus de la rougeole dans le sérum. Le test d'inhibition de l'hémagglutination, utilisé fréquemment dans le passé, s'est avéré peu sensible et inadapté pour un grand nombre d'échantillons. Les tests ELISA quant à eux, sont en général plus rapides et plus sensibles. Cependant les taux d'anticorps obtenus par ELISA ne donnent pas nécessairement une bonne corrélation avec le niveau de protection [Cohen et al. 2005]. En effet, seuls les anticorps neutralisants dirigés contre les protéines H et F protègent contre une infection [De Swart et al. 2005]. Ainsi la corrélation des résultats par ELISA avec le niveau de protection dépend fortement de l'antigène utilisé pour mesurer les anticorps [Hartter et al. 2000]. La majorité des tests ELISA commercialisés utilisent du virus complet comme antigène et ne reflètent donc pas directement le niveau de protection. Le test de neutralisation de plaques est aussi un test très sensible et il mesure cette fois spécifiquement le taux d'anticorps neutralisants. Cependant ce test requiert un laboratoire adapté pour réaliser des cultures cellulaires sur une durée minimale de 5 jours.
Chen et collègues ont suggéré qu'un taux d'anticorps inférieur à 120 milli unités internationales en test de neutralisation de plaques est insuffisant pour protéger contre la maladie [Chen et al. 1990]. Des travaux plus récents suggèrent que des taux d'anticorps plus élevés sont nécessaires pour protéger les individus contre une infection silencieuse ou une forme atténuée de la rougeole [Muller et al. 1996; Huiss et al. 1997].

IV.5.2. Estimations de la durée de protection après vaccination

En général on distingue entre deux types d'études pour étudier la durée de protection après vaccination : (i) les études cross-sectionales et (ii) les études longitudinales.

Dans le cadre d'études cross-sectionales, on détermine les taux d'anticorps chez des individus d'âges différents en même temps. En comparant les taux d'anticorps dans les différents groupes d'âges, on peut alors déterminer l'évolution des taux d'anticorps après vaccination. Par exemple, Mossong et collègues ont comparé les taux d'anticorps chez 1141 enfants canadiens, 3 à 15 ans après vaccination. Dans cette étude, une perte moyenne de 5,6% des anticorps par an a été estimée en comparant les taux d'anticorps au temps après vaccination [Mossong et al. 2000]. Une autre étude cross-sectionale menée au Taiwan a comparé les taux d'anticorps 1 à 6 ans après vaccination. Dans cette étude la perte moyenne d'anticorps par an était supérieure à 30%, ce qui pourrait indiquer une perte d'anticorps accélérée pendant les premières années suivant la vaccination [Lee et al. 2001].

Dans le cadre d'enquêtes longitudinales les taux d'anticorps sont déterminés à plusieurs reprises avant et/ou après vaccination chez les mêmes individus. Ceci permet de suivre l'évolution des taux d'anticorps pour chaque personne individuellement. De telles études sont particulièrement intéressantes pour analyser l'effet d'une revaccination ou d'un contact avec le virus sauvage. Par exemple, Davidkin et collègues ont pu montrer que l'augmentation des taux d'anticorps après revaccination était plus fréquente et plus importante lorsque le niveau d'anticorps avant la deuxième dose était bas [Davidkin et Valle 1998].

L'évolution des anticorps antirougeole a été analysée dans de nombreuses études cross-sectionales et longitudinales. En résumé les résultats les plus importants étaient :

(i) Les anticorps contre la rougeole, induits par vaccination, restent longtemps (>15 ans) détectables chez la plupart des vaccinés

(ii) Les taux d'anticorps diminuent après vaccination et les taux de diminution semblent accélérés pendant les premières années suivant la vaccination

(iii) L'augmentation des taux d'anticorps par revaccination d'individus séropositifs est de courte durée

(iv) L'évolution des anticorps anti-rougeole est influencée par la fréquence de contact avec des souches sauvages du virus.

V. Epidémiologie moléculaire du virus de la rougeole

L'épidémiologie moléculaire du virus de la rougeole est un outil important pour la surveillance de la maladie. Basée sur l'analyse génétique des souches virales circulant dans différentes régions à différents moments, l'épidémiologie moléculaire du virus de la rougeole permet de :

(i) suivre les voies de transmission du virus
(ii) documenter une interruption de la transmission du virus
(iii) évaluer l'efficacité des programmes de vaccination
(iv) analyser des cas de rougeole associés à la vaccination

V.1. Classification génétique du virus de la rougeole

Bien que monotypique du point de vue sérologique le virus de la rougeole a une variabilité génétique considérable. De nombreux variants ont évolué grâce au taux d'erreurs élevé de l'ARN polymérase lors de la réplication virale [Rima et al. 1997; Schrag et al. 1999]. Ainsi, la variabilité nucléotidique atteint 7% pour les gènes les plus variables, H et N [Mulders et al. 2001]. Dans la partie C-terminale du gène N la diversité génétique entre deux souches sauvages peut même être supérieure à 13%.

Cette variabilité génétique entre les différentes souches sauvages a permis de les classer dans des groupes génétiques. En 1998 l'OMS avait établi un premier protocole standardisé pour la classification génétique des différentes souches de la rougeole [WHO - World Health Organization 1998]. On distinguait 8 clades, désignés par les lettres A à H, eux-mêmes subdivisés en 15 génotypes (A, B1-B2, C1-C2, D1-D6, E, F, G, H). De plus, une souche de référence, en général le premier isolat obtenu, a été désignée pour chaque génotype. D'après ce protocole le génotype d'une nouvelle souche devait être déterminé par séquençage complet du gène H ou des 450 nucléotides finaux de la partie C-

terminale du gène N. Le génotype est alors déterminé par analyse phylogénétique, par comparaison avec les souches de référence (Figure 6). Une différence minimale de 2.5% pour la partie C-terminale du gène N et de 2% pour le gène H entier par rapport à la souche connue la plus étroitement apparentée est requise pour la désignation d'un nouveau génotype.

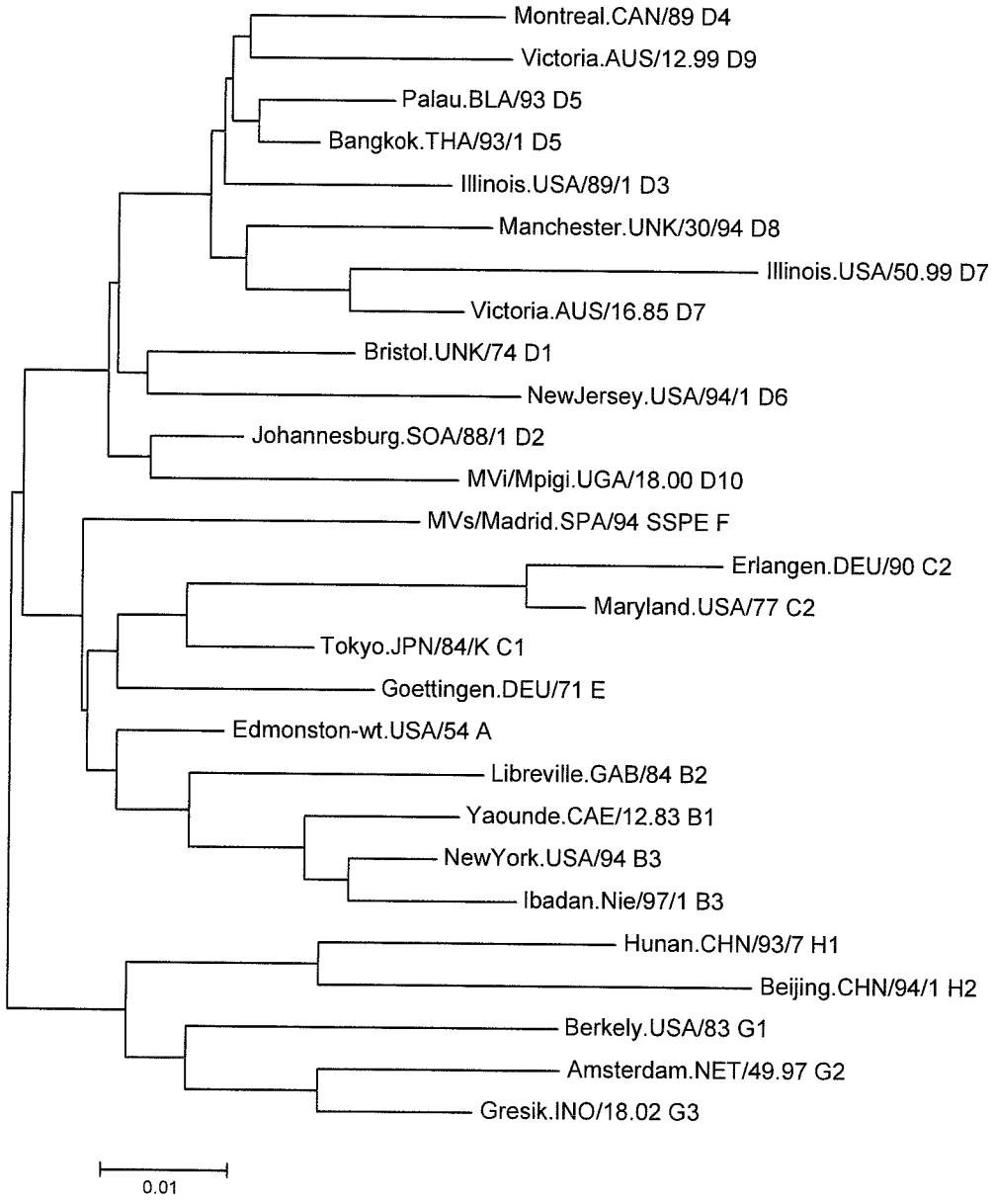

Figure 6 : Distance génétique entre les souches de références des différents génotypes du virus de la rougeole, sur la base des 450 nucléotides finaux de la partie C-terminale du gène N.

Depuis 1998, la liste des génotypes est passé de 16 à 23 (A, B1-B3, C1-C2, D1-D10, E, F, G1-G3, H1-H2) (Tableau 1). Cependant cinq génotypes (B1, D1, E, F, G1), qui n'ont pas été détectés depuis plus de 15 ans, ont été classés comme génotypes inactifs [WHO - World Health Organization 2005].

Tableau 1 : Souches de référence utilisées pour l'analyse phylogénétique des souches sauvages du virus de la rougeole [WHO - World Health Organization 2005]

Génotype	Etat	Souches de référence	Numéro d'accès à Genbank	
			Gène H	Gène N
A	Actif	Edmonston-wt.USA/54	U03669	U01987
B1	Inactif	Yaounde.CAE/12.83 "Y-14"	AF079552	U01998
B2	Actif	Libreville.GAB/84 "R-96"	AF079551	U01994
B3	Actif	New York.USA/94	L46752	L46753
		Ibadan.NIE/97/1	AJ239133	AJ232203
C1	Actif	Tokyo.JPN/84/K	AY047365	AY043459
C2	Actif	Maryland.USA/77 "JM"	M81898	M89921
		Erlangen.DEU/90 "WTF"	Z80808	X84872
D1	Inactif	Bristol.UNK/74 (MVP)	Z80805	D01005
D2	Actif	Johannesburg.SOA/88/1	AF085198	U64582
D3	Actif	Illinois.USA/89/1 "Chicago-1"	M81895	U01977
D4	Actif	Montreal.CAN/89	AF079554	U01976
D5	Actif	Palau.BLA/93	L46757	L46758
		Bangkok.THA/93/1	AF009575	AF079555
D6	Actif	New Jersey.USA/94/1	L46749	L46750
D7	Actif	Victoria.AUS/16.85	AF247202	AF243450
		Illinois.USA/50.99	AY043461	AY037020
D8	Actif	Manchester.UNK/30.94	U29285	AF280803
D9	Actif	Victoria.AUS/12.99	AY127853	AF481485
D10	Actif	Kampala.UGA/51.00/1	AY923213	AY923185
E	Inactif	Goettingen.DEU/71 "Braxator"	Z80797	X84879
F	Inactif	MVs/Madrid.SPA/94 SSPE	Z80830	X84865
G1	Inactif	Berkeley.USA/83	AF079553	U01974
G2	Actif	Amsterdam.NET/49.97	AF171231	AF171232
G3	Actif	Gresik.INO/17.02	AY184218	AY184217
H1	Actif	Hunan.CHN/93/7	AF045201	AF045212
H2	Actif	Beijing.CHN/94/1	AF045203	AF045217

Certains clades, comme le clade A, ne comportent qu'un seul génotype tandis que d'autres, comme le clade D, sont représentés par de nombreux génotypes. Certains génotypes ont été détectés fréquemment (par exemple D6) tandis que d'autres sont rarement apparus (par exemple E,F). Certains génotypes sont associés à une certaine région géographique, tandis que d'autres sont distribués beaucoup plus largement. Ainsi les virus du clade B prédominent dans les régions sub-sahariennes et centrales de l'Afrique. Les virus des clades G et H dominent en Asie du Sud-Est et en Chine. Par contre les différents génotypes du clade D sont distribués de façon ubiquitaire sur différents continents [Muller et Mulders 2002].

V.2. L'épidémiologie moléculaire comme outil de surveillance de la rougeole

V.2.1. Identification des voies de transmission du virus

Le séquençage de souches sauvages isolées à différents endroits et/ou à différents moments, permet de mieux caractériser les voies de transmission du virus. On distingue principalement deux modes de transmission :

(i) la transmission continue (endémique) à l'intérieur d'une population
(ii) la transmission (importation) du virus d'une population à une autre

L'identification répétée d'un même génotype au sein d'une population, indique que le virus a pu établir des chaînes de transmission continues parmi ses membres. Dans le cadre d'une épidémie, cette circulation du virus est d'une durée limitée. Lorsque la circulation du virus persiste pendant plusieurs années, on considère que la circulation est endémique.
La variabilité nucléotidique entre différentes souches virales détectées dans une même région permet d'estimer le nombre de chaînes de transmission

indépendantes ainsi que leur durée d'existence. Il est ainsi possible de distinguer une situation endémique d'une épidémie. Ainsi l'identification de nombreuses variantes du génotype B3 au Nigeria en 1997/1998 (diversité génétique dans la partie C-terminale du gène N >4.2%) indiquait l'existence prolongée de nombreuses chaînes de transmission indépendantes, mettant en évidence la circulation endémique du virus dans ce pays [Hanses et al. 1999]. Une situation comparable a aussi été rencontré en Chine (1993-1994, [Xu et al. 1998]).

Par contre différentes souches virales isolées au cours d'une épidémie de durée limitée sont en général génétiquement très semblables. Ainsi la variabilité génétique parmi des souches isolées pendant une épidémie au Luxembourg était inférieure à 0.2 % [Hanses et al. 2000]. Les épidémies de rougeole sont en général déclenchées par l'introduction du virus dans une population insuffisamment protégée. Dans la plupart des cas le virus est importé d'une région où la circulation du virus est restée endémique. L'identification du génotype du virus importé peut aider à identifier la source d'importation [Rota et al. 2001].

Au cours des dernières années, des efforts considérables ont été faits pour identifier les génotypes des souches sauvages circulant dans les régions avec transmission endémique, principalement en Afrique et en Asie du Sud-Est [Riddell et al. 2005]. Cette cartographie a permis d'identifier ou de confirmer la source d'importation de nombreux cas de rougeole dans des régions où la circulation du virus avait été interrompue. Aux Etats-Unis par exemple, une grande variété de génotypes a été détectée depuis l'interruption de la circulation prolongée du génotype D3 en 1992. Dans la majorité des cas, les sources d'importation ont pu être déterminées avec l'aide de l'épidémiologie moléculaire [Rota et al. 1996; Rota et al. 2001]. D' autre part la caractérisation génétique de certaines souches dont la source d'importation était connue a permis d'identifier le génotype circulant dans d'autre pays avant que les premières séquences n'y aient été caractérisées. Ainsi une souche importé depuis l'Indonésie aux Pays-Bas attestait la circulation du génotype G2 dans cette région avant l'identification

des premières séquences provenant d'isolats obtenus directement dans la région [De Swart et al. 2000].

V.2.2. Identification d'interruption de la circulation du virus

La caractérisation régulière de souches sauvages détectées dans une même région permet de déterminer si la circulation du virus parmi les membres de la population était continue ou interrompue. L'absence prolongée d'un génotype identifié auparavant indique que la chaîne de transmission de ce virus a été interrompue. Ainsi l'absence du génotype C1 au Japon depuis le début des années 1990, en dépit de sa dominance pendant la décennie précédente, indique que la circulation des virus appartenant à ce génotype a été interrompue [Katayama et al. 1997]. Egalement l'absence prolongée de souches virales apparentées au génotype D3, responsable de la dernière épidémie importante aux Etats-Unis, démontre que toutes les chaînes de transmission de ce génotype ont pu être interrompues [Rota et al. 1996]. Ainsi l'épidémiologie moléculaire peut aussi permettre de distinguer entre une circulation continue du virus et une suite d'épidémies consécutives, lorsque celles-ci résultent d'importations indépendantes de souches suffisamment différentes. L'illustration d'une interruption de transmission du virus est d'autant plus importante dès lors qu'il faut démontrer l'efficacité d'une stratégie de vaccination.

V.2.3. Efficacité des programmes de vaccination

Avant l'introduction de la vaccination, la circulation du virus de la rougeole était endémique sur tous les continents. Différents génotypes dominaient dans différentes régions du monde. Entre temps de nombreux pays ont pu interrompre la circulation du virus au moins temporairement. Bien que des analyses épidémiologiques classiques aient permis de démontrer une diminution des cas de rougeole après vaccination, il est difficile de démontrer l'interruption complète de la transmission du virus par ces moyens. Voilà pourquoi les études

d'épidémiologie moléculaire ont été importantes pour démontrer que la transmission du virus endémique pouvait être interrompue par l'application d'une certaine stratégie de vaccination.
De plus l'épidémiologie moléculaire peut indiquer un progrès vers le contrôle du virus grâce à la vaccination, même si la transmission endémique n'est pas complètement interrompue. Ainsi, seulement deux souches différentes co-circulaient après une campagne de vaccination au Burkina Faso [Mulders et al. 2003]. Vu le grand nombre de variantes qui co-circulaient dans certains pays voisins, ces observations indiquaient un progrès important vers le contrôle de la maladie.

V.2.4. Cas de maladies associées à la vaccination

Le séquençage partiel de l'ARNv permet aussi de distinguer entre souches vaccinales et souches sauvages. Les souches vaccinales appartiennent toutes au génotype A et sont génétiquement quasi identiques, bien qu'elles soient dérivées de différentes souches sauvages [Bellini et al. 1994]. Ainsi tous les cas de rougeole associés à un autre génotype (non A) sont clairement dus à une infection avec une souche sauvage. Par contre l'isolation de souches virales appartenant au génotype A doit être analysée avec prudence. Bien que la circulation de souches sauvages appartenant au génotype A ne puisse être totalement exclue, ceci est peu probable. Des souches appartenant au génotype A ont été détectées à plusieurs reprises sur le continent américain, en Asie et en Europe [Bellini et al. 1994; Rota et al. 1994; Outlaw et Pringle 1995; Xu et al. 1998]. Cependant la similitude génétique des isolats obtenus pendant une période de >40 ans dans différentes régions est difficile à concilier avec une circulation continue de souches sauvages appartenant à ce génotype. Voilà pourquoi les cas de rougeole associés au génotype A seraient plus facilement expliqués par la maladie après vaccination. Cette hypothèse est souvent supportée par l'identification de séquences identiques ou quasi-identiques à celles des souches vaccinales. Cependant les effets secondaires à la vaccination

contre la rougeole sont rares, bien qu'elle puisse déclencher une rougeole chez des personnes sévèrement immunodéprimées. Puisque les souches vaccinales sont souvent utilisés comme contrôle positif dans la plupart des laboratoires, la détection d'une souche appartenant au génotype A doit être interprétée correctement tout en excluant une contamination.

RÉSULTATS ET DISCUSSION

VI. Déclin d'anticorps après vaccination contre la rougeole et la rubéole – une étude longitudinale.

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Résumé de l'article

Plusieurs études ont montré que les vaccins vivants atténués pour la rougeole et la rubéole sont très efficaces en terme de séroconversion, même après l'administration d'une seule dose. Cependant les taux d'anticorps semblent diminuer avec le temps après vaccination. Aujourd'hui une grande partie des populations est toujours protégée par immunité naturelle après infection avec le virus sauvage. Cependant la proportion d'individus vaccinés augmente continuellement. Voilà pourquoi il est important d'étudier la durée de protection conférée par vaccination dans le cadre des programmes de contrôle des deux virus. Le but de cette étude longitudinale était d'estimer la demi-vie des anticorps contre la rougeole et la rubéole après vaccination ainsi que l'effet d'une seconde dose sur les taux d'anticorps.

Les taux d'anticorps ont été déterminés par ELISA (Enzygnost, Dade-Behring, Marburg, D) chez 224 jeunes adolescents vaccinés, de trois écoles secondaires différentes au Luxembourg entre 1996 et 2003. Un premier sérum a été obtenu à l'âge de 11.8 à 15.1 ans, le deuxième 6.8 ans plus tard. La plupart des participants avait reçu une première dose de vaccin à virus atténué avant l'âge de 5 ans (rougeole (RO): 92.5% ; rubéole (RU) 82.7%). La majorité avait reçu une deuxième dose 1 à 60 mois avant ($VV0_{RO}$: 22.1% ; $VV0_{RU}$: 21.0%) ou 0.1 à 6.6 ans après ($V0V_{RO}$: 52.6% ; $V0V_{RU}$: 49.9%) le prélèvement du premier sérum. Les autres n'ont jamais été vaccinés (000_{RO} : 7.5% ; 000_{RU} : 17.3 %) ou n'ont reçu qu'une seule dose jusqu'à la fin de l'enquête ($V00_{RO}$: 22.1% ; $V00_{RU}$: 21.0%).

Pour la rougeole une diminution des taux de séroprévalence a été observée chez les individus non revaccinés entre les deux prélèvements (-4.2 à -7.9%) alors que la séroprévalence contre la rubéole était plus stable (0 à -2%) dans les mêmes groupes. Parmi les participants revaccinés pendant la période de l'enquête (V0V), la séroprévalence contre la rougeole est resté stable (environ 90%). Par contre une augmentation du taux de séroprévalence contre la rubéole, de 92.1 à 100%, fut déterminée dans la même catégorie.

Plusieurs participants séropositifs avaient des taux d'anticorps significativement supérieurs (> 3 fois) dans le deuxième sérum (RO : n=10 ; RU n=10), bien que seulement une partie d'entre eux avait été revaccinée après le premier prélèvement (RO : n = 5 ; RU : n = 7). Tous les individus concernés avaient des taux d'anticorps relativement bas dans le premier sérum (RO : <1500 mUI ; RU <40 UI). Au contraire, des taux d'anticorps significativement diminués ont été observés chez des individus (RO : 6 ; RU : 6) ayant des taux plus élevés dans leur premier sérum (RO : >3500 mUI ; RU : >100 mUI).

L'évolution des anticorps a été évaluée davantage, parmi les individus n'ayant pas subi un changement significatif des taux d'anticorps, en comparant les moyennes géométriques des taux d'anticorps (MG) obtenus dans les différentes catégories de vaccination. Parmi les individus non revaccinés pendant la période de l'enquête, une diminution significative ($p<0.05$) (RO : -11.7 à -14.7 ; RU -19.6 à -30.8% ; $p<0.05$) de la MG fut déterminée pour la rougeole (V00 : -11.7% ; VV0 : -14.7%) ainsi que la rubéole (V00 : -19.6% ; VV0 : -30.8%). Bien que statistiquement non significative, une diminution de la MG des taux d'anticorps contre la rougeole a aussi été observée dans la catégorie V0V (-6.5% ; $p=0.07$), alors que les taux d'anticorps contre la rubéole avaient tendance à augmenter dans le même sous-groupe (+11.1% ; $p=0.07$). Par ailleurs, une diminution importante (RO : -16.4% ; RU : -15.5%) des anticorps fut observée parmi tous les individus avec des taux de départ élevés (RO : >1500 mUI ; RU : 40 mUI). Au contraire les taux d'anticorps étaient relativement stables chez les participants avec des taux plus bas dans leur premier sérum, indépendamment des différentes sous-catégories de vaccination.

La présente enquête confirme donc, que les taux d'anticorps contre la rougeole et la rubéole diminuent après vaccination avec des vaccins à virus vivants atténués. Bien que la perte d'anticorps contre la rubéole semble accélérée, l'efficacité de stimulation par une deuxième dose était plus efficace par rapport à la rougeole. Pour les deux virus, les taux de diminution étaient le plus important parmi les individus revaccinés juste avant le début de

l'enquête, ce qui indique que les anticorps stimulés disparaissent plus rapidement. L'augmentation significative des taux d'anticorps chez une partie des participants avec des taux de départ relativement bas, ne fut que partiellement expliquée par l'application d'une deuxième dose de vaccin pendant l'enquête. La stimulation d'anticorps en absence de revaccination serait le plus facilement expliquée par un contact avec le virus sauvage, bien que la circulation des deux virus au Luxembourg était très limitée pendant la période de l'enquête, confirmée par la présence d'individus séronégatifs, non vaccinés, à la fin de l'enquête.

Cependant l'augmentation significative et prolongée des taux d'anticorps, induite par le vaccin ou le virus sauvage, indique que ces individus, bien que séropositifs, n'étaient pas nécessairement protégés contre une infection par le virus. Le virus sauvage de la rougeole étant le virus le plus infectieux connu, parmi ceux qui sont transmis par aérosol, un maximum d'individus devra être protégée contre l'infection pour achever l'éradication globale du virus. La perte d'anticorps accélérée, lorsque les taux de départ sont élevés, pourrait rapidement augmenter la proportion d'individus faiblement protégés. Voilà pourquoi il sera important dans le futur de déterminer les taux d'anticorps nécessaires pour protéger contre toute infection par le virus sauvage même si elle s'avère cliniquement silencieuse. Puisque les individus avec des taux d'anticorps très bas pourraient aussi être protégés par des cellules T, il sera important d'analyser aussi la partie cellulaire du système immunitaire.

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Waning antibodies in measles and rubella vaccinees—a longitudinal study

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Abstract

The evolution of measles- and rubella-specific serum IgG was followed in a longitudinal study in 224 young adolescent vaccinees, with or without boost vaccination before or during the 6.8-year observation period. Antibody titres were monitored by enzyme immuno assay (Enzygnost®, Dade-Behring). After revaccination (second dose) rubella seropositivity rate increased from 92.1 to 100%, whereas measles seroprevalence (about 90%) did not significantly change between the paired sera. Significantly higher IgG (>three-fold) in the second serum of 5.2% (measles) and 7.8% (rubella) of participants with low antibodies (measles: <1500 mIU; rubella <40 IU) in first serum, suggest a secondary immune response (SIR) during the study period, only partially explained by revaccination. Excluding individuals with SIR, minimal annual antibody decay rates of −2.9% (confidence interval, CI: −0.7 to −4.8%) for rubella and −1.6% (CI: −0.1 to −3%) for measles were determined in participants with single dose vaccination. Thus, two-dose vaccination was adequate to protect women from rubella infection at least during childbearing age. Similarly only few individuals may become seronegative for measles again after successful vaccination due to minimal waning of low antibody levels (<1500 mIU). However, as a result of a more rapid decay of high-titre (>1500 mIU) antibodies (−2.4%/year), many vaccinees may eventually become susceptible to vaccine-modified measles (VMM) and consequently complicate measles control strategies.

Keywords: Measles and rubella IgG; Vaccination; Follow-up

1. Introduction

Comprehensive vaccination has dramatically decreased measles morbidity and mortality, both in developing countries and the developed world [1]. This has encouraged the World Health Organization to develop a strategic plan to eliminate measles in some of the global regions (the Americas, Europe and the Eastern Mediterranean region) and to further reduce measles mortality in Africa and Asia. Some protagonists even promote global eradication of measles [2,3].

Various strategies were implemented to optimize vaccination coverage. Most industrialized countries use a two-dose schedule with a first dose given at the age of 12–18 months and a second dose during earlier or later childhood, to compensate for primary or secondary vaccine failure after first dose. Such reinforced vaccination schedules have largely controlled measles virus (MV) transmission in many countries and have effectively interrupted circulation of indigenous virus in most of the Americas and a number of other countries [4–6]. In Luxembourg, measles vaccination of young children became routine in 1986 when vaccines were made freely available by Ministry of Health. Today a first dose is recommended at 15–18 months and a second dose at 5–6 years of age. Only few unrelated measles and rubella cases were reported in Luxembourg during the past 10 years, suggesting very low wild-type virus circulation in the country.

However, control of virus transmission still relies to a large extend on individuals with natural immunity acquired during childhood, which is well documented to last lifelong [7]. Although successful vaccination has proven to provide

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long-lasting protection against disease [8], the duration and robustness of vaccine-immunity is not fully understood and measles outbreaks have been reported from highly vaccinated communities [9,10]. Similarly specific antibody waning was also reported from rubella vaccinees [11,12]. Although rubella is a rather mild disease, infection during early pregnancy may lead to serious congenital birth defects [13]. Therefore, vaccination against rubella has been included in the national immunization programmes of many countries, to protect women against rubella disease, at least during their reproductive age.

Waning of measles and rubella antibodies has been investigated in a number of cross-sectional and few longitudinal studies [8,10–12,14–22] mostly by haemagglutination inhibition assays (HIA). Cross-sectional studies compare single values of titres of many individuals at different time points after vaccination. Such studies do not reveal heterogeneity in rates of antibody waning, and individual and environmental effects are confounded. The aim of the present study was to investigate in a longitudinal fashion with a sensitive enzyme immuno assay (EIA) the 6–7 year evolution of measles and rubella antibodies in paired sera of vaccinated individuals and to assess the effect of revaccination on antibody levels in the context of low wt MV and rubella virus (RV) circulation.

2. Materials and methods

2.1. Cohorts

Paired sera were collected from 224 schoolchildren, attending three different secondary schools located in the North (Lycée du Nord, Wiltz; $n=53$), or in the city of Luxembourg (Lycée Schuman, $n=55$; and Lycée Fieldgen, $n=116$). Five hundred and twelve children were originally enrolled in 1996, but only 297 returned for the second sampling in 2003; 73 of the latter had unreliable vaccination records and were excluded from the study. Informed consent was obtained from all participants or their guardians. The study was approved by the local ethics committee and the responsible Ministries.

A first sample was drawn in September/October of their first year of high school (1996) when children were on average 12.9 years old (range: 11.8–15.1). The second serum was collected 6.8 (±0.2) years later, before the end of the last year of high school, during May–July 2003. Vaccination records were checked on both occasions. Individuals which received their first measles or rubella vaccination only shortly before or after the first sampling were excluded. The remaining were classified for each vaccine (measles and rubella) into four different subgroups according to whether they were never vaccinated (000), only vaccinated during early childhood (V00), or revaccinated before (VV0) or after (V0V) the first sampling (Table 1). The categories were used for both measles and rubella, but the measles (000_M, $V00_M$, $VV0_M$, $V0V_M$) and rubella (000_R, $V00_R$, $VV0_R$, $V0V_R$) cohorts overlapped only partially since 32.0% of vaccinations during early childhood did not contain the rubella component.

One hundred and ninety-seven participants were vaccinated between 9 and 60 months of age (89.9% between 12 and 36 months) with either trivalent (134/197) measles-mumps-rubella (MMR) (Pluserix®, GlaxoSmithKline, containing Schwarz measles, RA27/3 rubella and Urabe-9 mumps strain), divalent (40/197) measles-mumps (Rimparix®, GlaxoSmithKline, containing Schwarz and Jeryl Lynn strains) or monovalent (23/197) measles vaccine (Attenuvax®, Merck, containing Enders' Edmonston strain) (Table 1). Thirty-eight of them received a first ($n=14$) or second dose ($n=24$) of measles-mumps-rubella vaccine (MMRII®, Merck, containing Enders' Edmonston measles, RA27/3 rubella and Jeryl Lynn mumps strain) 1–20 months before the first sampling. One hundred and twelve received a first ($n=40$) or second ($n=72$) dose of MMR (MMRII®, Merck) during the study period, 6.8–0.2 years before the second sample was collected. Sixteen or 28 individuals were never vaccinated against measles or rubella.

The cross-sectional study was based on sera of 2673 individuals from Luxembourg of different age groups (4–82 years) [20]. Measles IgG antibody levels were determined by Enzygnost® as described below. Individuals were grouped into 19 age categories each one including 54–328 participants. GMTs as well as the percentages of participants with antibody levels <1500 or <250 mIU were determined for each age group (Fig. 4).

2.2. Antibodies

Peripheral blood was obtained by venipuncture and incubated for 1 h at 37 °C. Serum was collected after centrifuga-

Table 1
Numbers of participants by categories of their vaccination status with respect to measles or rubella

Group	First vaccination year	Second vaccination year	Measles N^a (%)	Rubella N^a (%)
000	–	–	16 (7.5)	28 (17.3)
V00	1983–1988	–	47 (22.1)	34 (21.0)
VV0	1983–1988	1992–1996[b]	38 (17.8)	24 (14.8)
V0V	1983–1988	1996[c]–2003	112 (52.6)	76 (46.9)
Total			213 (100)	162 (100)

[a] Number of individuals.
[b] Before first sampling in 1996.
[c] After first sampling in 1996.

tion for 25 min at 1700 × *g*, divided into aliquots and stored at −20 °C until testing.

Anti-MV and anti-RV IgG levels were assessed using commercial enzyme immuno assays (Enzygnost® Anti-Measles-Virus IgG and Enzygnost® Anti-Rubella-Virus IgG, Dade-Behring, Marburg, Germany), based on antigens derived from cell culture grown virus. For both measles and rubella testing, all sera were included together in a single experiment and recommended incubation times were strictly followed. Paired sera of the same individuals were tested in the same microtiter plate and all plates belonged to the same batch. Following the manufacturers instructions, sera were considered anti-MV or anti-RV IgG positive if the corrected absorbance was higher than 200 m OD and negative when lower than 100 m OD. Absorbance values between 100 and 200 m OD were considered as equivocal. Antibody titres were calculated for all sera with a corrected absorbance >100 m OD and uncorrected absorbance ≤2500 m OD, using the alpha-method [23] according to the manufacturer's guidelines. The antibody titres were based on International Standards for Anti-Measles (1st international standard preparation) or Anti-Rubella (2nd international standard preparation) sera of WHO, included as positive controls in the kit. Titres were calculated using the equation log 10 titre = αOD^{β}, where α and β are two batch specific constants provided by the manufacturer. According to the manufacturer, sensitivity and specificity of the measles (or rubella) assay are 99.6% (or 100%) and 100% (or 98.5%), respectively. Statistical significance between geometric mean titres (GMT) and 95% confidence intervals (CI) were calculated using SigmaStat version 3.0.1a software (Systat software Inc., Point Richmond, US) with two-tailed paired Student's *t*-test after confirming normal distributions of individual titres (log mIU/ml).

The annual waning rate was derived from the decrease of GMTs over the 6.8-year study period. The waning rate in participants with titres >1500 mIU in the first serum was used to estimate the number of participants which would fall below (i) 250 mIU (threshold for seropositivity) or (ii) 1500 mIU within 70 years following 2003.

3. Results

3.1. Seroprevalence

Two third of unvaccinated participants were measles IgG positive in their first serum, suggesting contact with wild-type virus before 1996 (Table 2). The 1996-seronegatives were from all three schools and did not seroconvert through 2003, suggesting that wild-type MV was absent in these communities throughout the study period. In contrast, rubella seroprevalence among unvaccinated participants increased from 39.3 to 50.0% during the study period. Two participants from Lycée Fieldgen and one from Lycée du Nord seroconverted most probably due to contact with wt rubella virus. However, 14 unvaccinated individuals did not seroconvert between samplings, therefore also rubella virus circulation was considered very low in the study cohorts.

Seroprevalence of specific IgG among all measles vaccinees decreased from 91.4 to 89.3% between 1996 and 2003 (Table 2). A decrease in seroprevalence rates was observed among vaccinees whether they were vaccinated once (group V00: −4.2%) or twice (group VV0: −7.9%) before the first sampling (Table 2). The reduced seroprevalence was due to two (V00) or three (VV0) individuals that were classified equivocal in 2003 after having had weak positive absorbance values (<370 m OD) in 1996. In contrast seroprevalence did not decrease in VOV group. Despite revaccination 1–6 years before the second sample was collected, 9.8% of participants had no protective antibody levels in their second serum. Transient seropositivity during the study period can of course not be excluded.

Anti-RV seroprevalence among all rubella vaccinees increased from 94.6 to 98.0% during the study period (Table 2). Seroprevalence increased from 92.1 to 100% among participants that were revaccinated between samplings (V0V). In the VV0 group all participants were seropositive at both time points. One V00 participant, seropositive in the first serum, was classified equivocal at the end of study.

Thus, rubella seroprevalence significantly increased among vaccinees during the study period, due to revaccination of seronegative participants, whereas measles seropreva-

Table 2
Measles and rubella seroprevalence (in 1996 and 2003) among participants grouped according to vaccination status (see Table 1)

Group	N^a	Measles seroprevalence %		N^a	Rubella seroprevalence %	
		1996	2003		1996	2003
Unvaccinated group						
000	16	62.5	62.5	28	39.3	50.0
Vaccinated groups						
V00	47	93.6	89.4	34	95.8	93.8
VV0	38	94.7	86.8	24	100.0	100.0
V0V	112	89.3	90.2	76	92.1	100.0
Total	197	91.4	89.3	134	94.6	98.0

[a] Numbers of participants.

lence somewhat decreased despite revaccination of >50% of participants. At the end of the study all participants with two-dose regimen were seropositive for rubella (confirming the high efficacy of the rubella vaccine), in contrast to 16/150 participants which had only greyzone or negative measles antibody levels in their second serum despite two-dose vaccination and must therefore be considered as primary or secondary vaccine failures.

3.2. Waning of antibodies in seropositive vaccinees

According to the EIA manufacturer, three-fold differences in anti-MV and anti-RV IgG titres are considered significant. Antibody titres (in IU) could only be determined in paired sera of 192 (anti-MV IgG) and 128 (anti-RV IgG) vaccinees because of an uncorrected absorbance above 2500 m OD in the others (see Section 2). Fig. 1 shows the ratios of titres in the paired sera (2003/1996) in both assays. Ratios >1 indicate an increase, ratios <1 a decrease in titres between 1996 and 2003.

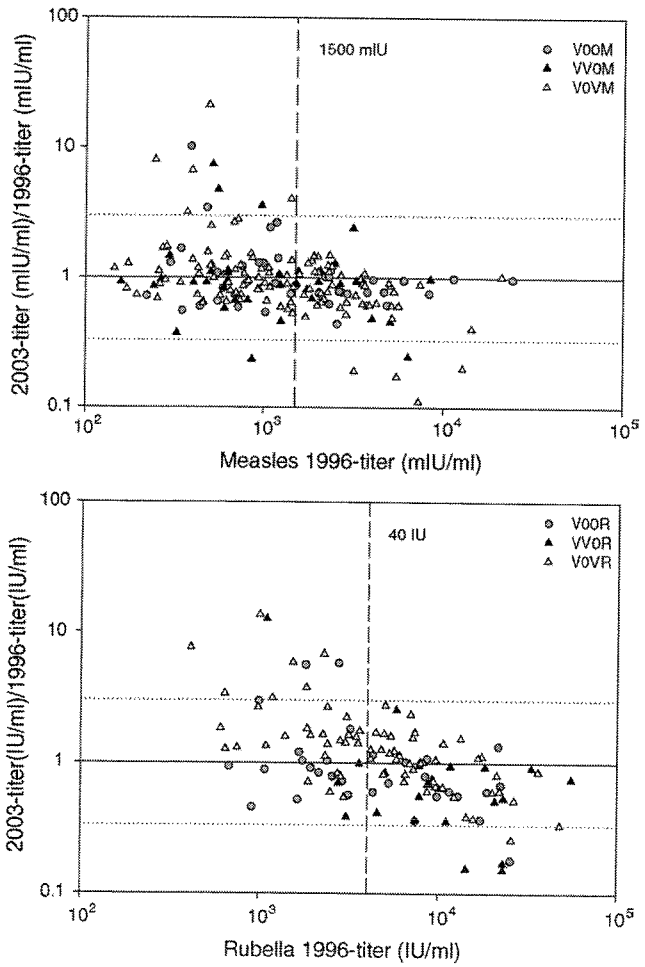

Fig. 1. Ratios of 2003- and 1996-titres (rubella or measles serum IgG) of vaccinees without (V00) or with revaccination before (VV0) or after (V0V) the first sampling plotted against the 1996 antibody titre. Dotted lines correspond to three-fold increase or decrease in antibody titres. Vertical dashed lines highlight the 1500 mIU (measles) or 40 mIU (rubella) threshold values.

A significant increase in anti-MV IgG was found in 10 vaccinated participants (5.2%), all of which had 1996-titres below 1500 mIU/ml. Half of them were revaccinated during the study period. Six individuals (3.1%) showed a significant decrease in anti-MV IgG titre within the 6.8 years of observation. Five of them had 1996-titres above 3500 mIU. Two of the six were revaccinated 3 and 9 months before the first sample was taken. Three of six subjects showing significant waning of anti-MV IgG may have been exposed to wild-type virus several months before the first serum was collected. Before entering one of the participating high schools, these individuals attended the primary schools, where the last major measles outbreak in Luxembourg occurred (February–June 1996) [24].

Anti-RV IgG was significantly increased in the second serum of 10 participants (7.8%), all of which had 1996-titres below 40 IU. Seven of them had been vaccinated during the study period. Six individuals (4.7%) showed a significant decrease in titre within the 6.8 years of observation. All of them had 1996-titres above 100 IU. Three of them were revaccinated less than 1.5 years before the first sample was obtained. In two cases (1 V0V and 1 V00) significant increases were detected for both measles and rubella antibodies, although only one of them received MMR vaccine between samplings. One individual (VV0) had significantly higher anti-RV IgG but significantly lower anti-MV IgG in the second serum.

These results suggest that both measles and rubella-specific antibodies may only be significantly boosted, when pre-boosting titres are below a certain threshold. Participants with a significant increase in antibody levels most probably developed a secondary immune response (SIR) during the study period, although only some of them were revaccinated after first sample collection. Moreover, a significant antibody boost was found in only 5/62 (anti-MV IgG) and 7/31 (anti-RV IgG) individuals with 1996-titres below threshold (anti-MV IgG <1500 mIU/ml or anti-RV IgG <40 IU/ml) and documented revaccination between samplings. It is possible that revaccination also induced a transient increase in measles or rubella IgG among other participants during the study period, which had already disappeared in the second serum. Also, significant antibody increase was not correlated with time since revaccination.

We also investigated the change in antibody levels among individuals without significant difference between paired sera. When sera of all subgroups were included a statistically significant decrease in GMT between paired sera was found only for measles IgG (−9.2%; $p < 0.001$). Although rubella IgG levels also tended to decrease, the difference in GMT was not significant ($p = 0.2$). Both measles and rubella-specific antibodies significantly ($p < 0.05$) decreased among participants, which were not revaccinated between samplings (Fig. 2): Moreover, the extent of antibody decay was greater for rubella than for measles within the groups V00 (−19.6% versus −11.1%) and VV0 (−30.8% versus −14.7%). For both viruses the largest and most significant loss of specific

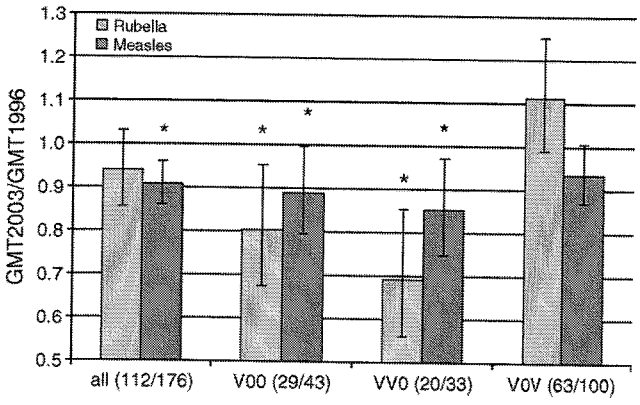

Fig. 2. The ratio of GMTs (±95% CI) of paired sera ($GMT_{2ndsera}$/$GMT_{1stsera}$) of seropositive vaccinees without (V00) or with revaccination before (VV0) or after (V0V) the first sampling. Number of participants (rubella/measles) in subgroups. Asterisks (*) indicate statistical significance ($p < 0.05$) of the difference by paired Student's t-test.

antibodies was found in VV0 group. Since these individuals were revaccinated shortly before the first sample was taken, this seems to reflect rapid waning after a boost vaccination (Fig. 2). In the VOV groups MV IgG levels tend to decrease (−6.5%; $p = 0.07$) despite revaccination, whereas anti-RV IgG tend to increase (+11.1%; $p = 0.07$) within the same group, but these differences were not significant (Fig. 2).

We also compared the fate of antibodies in vaccinees with low (measles IgG < 1500 mIU/ml; rubella IgG < 40 IU/ml) or high titres (measles > 1500 mIU/ml; rubella IgG > 40 IU/ml) in the first serum. These threshold values were chosen on the basis of the above findings, showing that all participants with significantly increased antibodies in their second serum had titres below these thresholds in the first serum. Interestingly, no significant difference in GMT was found for both measles ($p = 0.4$) and rubella ($p = 0.1$) in low titre participants. In contrast, a highly significant decrease of −16.4% (CI: −10.1 to −22.4%; $p < 0.001$) and −15.5% (CI: −5.5 to −24.1%; $p = 0.004$) was found for measles and rubella IgG in high-titre individuals. This may partly be due to a more efficient antibody boost in VOV participants with low antibody levels before revaccination: Measles GMT of VOV individuals with high antibody levels in first serum ($n = 43$) significantly decreased by 15.3% ($p < 0.001$), whereas no significant difference ($p = 0.6$) was found among those with low antibody levels ($n = 57$). Interestingly, rubella GMT even increased (36%, CI: 13.9–62.6%; $p = 0.002$) among low titre V0V participants ($n = 24$), and did not significantly change ($p = 0.8$) among those with high titres, further confirming the more efficient rubella antibody boost after revaccination as compared to measles.

A similar tendency was also observed among participants which were not revaccinated (V00), although their number was more limited ($n = 14$–23) in the high and low antibody subgroups. Antibodies significantly decayed among high titre V00 individuals (measles: −19.6%, CI: −10.7 to −27.7%, $p < 0.001$; rubella: −30.7%, CI: −12.9 to −44.9%, $p = 0.004$), whereas GMTs of the low titre subgroups did not significantly change (measles: $p = 0.7$; rubella: $p = 0.5$).

4. Discussion

We followed up humoral immunity against measles and rubella in schoolchildren from Luxembourg with or without revaccination before or during a 6.8-year study period. As expected from previous studies both measles and rubella-specific serum antibody levels decrease with time after vaccination. Minimal estimates of decay rates after single dose (V00) vaccination were almost twice as high for rubella (−2.9%/year; CI: −0.7 to −4.8%/year) than for measles (−1.6%/year; CI: −0.1 to −3%/year), when participants with significantly different (>three-fold) antibody levels were excluded. On the other hand, a second dose (V0V) increased rubella positivity from 92.1 to 100% whereas revaccination did not further increase the measles seroprevalence. Sixteen (10.6%) participants had measles antibody levels below seropositivity threshold (<200 m OD) 1–8 years after second dose. Thirteen of them had greyzone antibody levels suggesting perhaps secondary vaccine failures. The transient nature of boosted antibodies is also reflected in the similar or lower measles antibody levels in the second serum despite revaccination in the $V0V_M$ group. In contrast rubella antibody boost after second dose was prolonged in comparison to measles, as indicated by a higher GMT in the second serum of revaccinated participants ($V0V_R$). Thus, seroprevalence was higher for rubella than for measles at the end of study probably as a result of the higher seroconversion rate and the more efficient antibody boost in response to the second dose.

A few participants showed a significant increase in measles and/or rubella antibodies, but only about half of them were revaccinated during the study period. Antibody boost in absence of revaccination would best be reconciled with exposure to wt virus, although MV circulation in Luxembourg was very low with only few unrelated cases reported during the study period. Exposure to wt virus during travel or failure to report revaccination may be other explanations. We have shown before, that exposure to wt MV may induce a prolonged increase in specific antibodies compared to pre-exposure levels, even though half-life of boosted antibodies was relatively short [25]. Interestingly three of six subjects showing significant waning of anti-MV IgG attended primary schools, in which a major measles outbreak occurred a few months before the first sample was collected and may therefore also reflect rapid waning of boosted antibodies.

Vaccination against rubella was mainly implemented to prevent congenital rubella syndrome (CRS) cases, by providing women with rubella immunity outlasting at least their reproductive age. All participants from this study had reached childbearing age when the second serum was collected. Our results confirm the previously reported, high immunogenicity of RA 27/3 rubella strain included in all MMR vaccines of the present study. However, antibody levels below 15 IU were

previously shown to allow reinfection [26–28], and antibody waning seems accelerated in comparison to measles. Using the above waning rate (−2.9%/year) obtained from $V00_R$ group, susceptibility to infection (<15 IU) would increase from 8.1 to 27.7% within the next 30 years among participants, but only few of them would become seronegative (<4 IU, 3.4%). A second dose vaccination during late adolescence could further reduce the number of rubella susceptibles in childbearing age.

Vaccination against measles is expected to reduce mortality in developing countries and eventually interrupt MV circulation. Experience supported by seroepidemiological models has shown that 95% community immunity is necessary to interrupt MV circulation in a population [24]. In the present cohort only 87.9% of participants were measles seropositive in their second serum. In addition, there are some indications that MV may infect and even circulate in seropositive individuals [10]. Measles has been described in highly vaccinated populations [29,30] and even in vaccinees with prior documented seroconversion [9,30–33]. Secondary immune responses without clinical symptoms after contact of seropositive individuals with wild-type virus further suggest that subclinical infection may be possible without disease [34]. The present study suggests that titres up to 1500 mIU do not always protect against SIR. 60% of the vaccinees (V00, VV0, V0V) had antibody titres below this threshold in 2003, an increase of 2% from 1996. Therefore, an important question is how many vaccinees will become susceptible to measles or vaccine-modified measles (VMM) and when. Fig. 3 shows that only after about 70 years, 50% of seropositive vaccinees from this cohort would become seronegative (<250 mIU) even if the high waning rate (−2.4%/year) determined among high-titre individuals (>1500 mIU/ml in 1996) was applied. However, because of minimal waning in the low titer individuals perhaps only a small fraction of vaccinees will ever become seronegative again. This corroborates

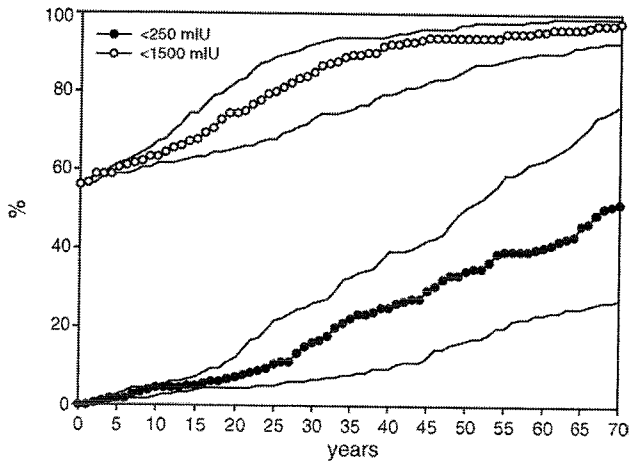

Fig. 3. Percent seropositive individuals of this study reaching antibody levels below 250 or 1500 mIU if 2.4% antibody waning/year is assumed. Year 0 corresponds to 2003. Lines without symbols correspond to percentages obtained when 95% CI values of the waning rate were used.

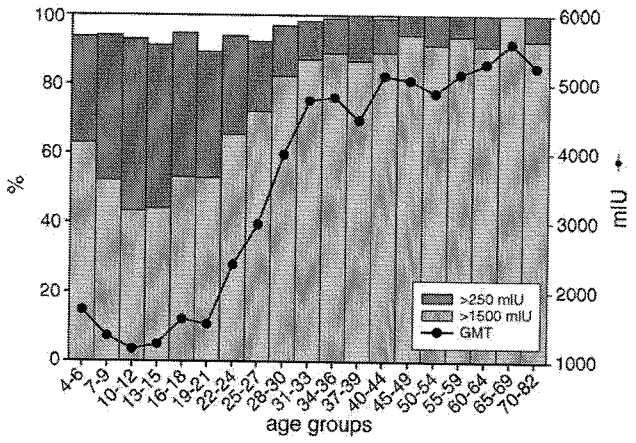

Fig. 4. Anti-MV IgG titres by age groups from a cross-sectional study in Luxembourg [20]. Percentages of individuals with titres >250 mIU (seropositives) and >1500 mIU (putatively protected from vaccine-modified measles) as well as the GMTs are shown.

observations in cross-sectional studies that the percentage of seronegatives is essentially constant (Fig. 4) in the vaccinated birth cohorts (4–24 years) irrespective of age or time after routine vaccination. However, an increasing proportion of vaccinees may become susceptible to subclinical infection (<1500 mIU/ml) associated with SIR and/or to a generally mild form of vaccine-modified measles (Fig. 3).

Anti-MV serum IgG levels from 2679 participants of a recent cross-sectional from Luxembourg (4–82 years of age) [20] are summarized in Fig. 4. GMT as well as the percentage of individuals with antibody levels <1500 mIU/ml first increases, then decreases again to very low levels with age of participants. Decreasing antibody levels in the early age groups most probably reflect waning antibodies after vaccination. Antibody mediated immunity increases with the proportion of convalescent patients in the higher age groups. As vaccinees gradually replace individuals with natural immunity, population immunity looses robustness because of failure to vaccinate, primary and secondary vaccine failures, as well as lower antibody titers and antibody waning in vaccinees in absence of wt virus circulation.

However, serum antibodies only partly correlate with protection against measles infection or disease. MV neutralizing antibodies target mainly the haemagglutinin protein [35] and to a lesser extend the fusion protein. The Enzygnost assay, however detects antibodies also against other MV proteins. Therefore, antibody titres determined by Elisa only partly correlate with MV neutralization titres [36]. Vaccinees can also be protected by T-cells, even if antibodies are low, and vaccine efficacy was shown to be higher after two doses of MMR during outbreaks, although antibody boost was of short duration [37,38]. How many vaccinees are really susceptible to reinfection and may eventually transmit wt virus must be further investigated in the context of measles outbreaks in highly vaccinated communities.

In summary, we have shown that 100% of participants were seropositive for rubella 1–8 years after second dose

MMR vaccination, despite waning antibodies, but <90% were seropositive for measles, indicating that measles antibody boost was less durable and immunity is likely to further erode as a result of waning antibodies. In vaccinees high titres of antibodies wane relatively fast and many of the vaccinees may become susceptible to vaccine-modified measles complicating measles control strategies. The more rapid antibody decay in the high titre groups may reflect their temporal proximity to boosting and their position on the antibody half-life decay curve. On the other hand, low titres apparently tend to be stable, suggesting that only few individuals may become seronegative again after successful vaccination. Both, boosted antibodies as a result of SIR and rapid waning of high-titer antibodies, which can only be detected in longitudinal studies, could considerably distort estimates of decay rates. Our results also suggest that mechanisms, which maintain antibodies, differ between measles and rubella after vaccination.

Acknowledgments

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References

[1] CDC. Update: Global measles control and mortality reduction—worldwide, 1991–2001. MMWR Morb Mortal Wkly Rep 2003; 52(20):471–5.
[2] de Quadros CA. Can measles be eradicated globally? Bull World Health Organ 2004;82(2):134–8 [Epub 2004 Mar 16].
[3] Orenstein WA, Strebel PM, Papania M, Sutter RW, Bellini WJ, Cochi SL. Measles eradication: is it in our future? Am J Public Health 2000;90(10):1521–5.
[4] Muller CP, Mulders MN. Molecular epidemiology of measles virus. In: Leitner T, editor. The molecular epidemiology of human viruses. Boston: Kluwer Academic Publishers; 2002. p. 237–72.
[5] Hanses F, van Binnendijk R, Ammerlaan W, Truong AT, de Rond L, Schneider F, et al. Genetic variability of measles viruses circulating in the Benelux. Arch Virol 2000;145(3):541–51.
[6] CDC. Progress toward measles elimination—region of the Americas, 2002–2003. MMWR Morb Mortal Wkly Rep 2004;53(14): 304–6.
[7] Panum P. Observations made during the epidemic of measles on the Faroe Islands in the year 1849. Med Classics 1938;3:829–86.
[8] Guris D, McCready J, Watson JC, Atkinson WL, Heath JL, Bellini WJ, et al. Measles vaccine effectiveness and duration of vaccine-induced immunity in the absence of boosting from exposure to measles virus. Pediatr Infect Dis J 1996;15(12):1082–6.
[9] Gustafson TL, Lievens AW, Brunell PA, Moellenberg RG, Buttery CM, Sehulster LM. Measles outbreak in a fully immunized secondary-school population. N Engl J Med 1987;316(13): 771–4.
[10] Pedersen IR, Mordhorst CH, Glikmann G, von Magnus H. Subclinical measles infection in vaccinated seropositive individuals in arctic Greenland. Vaccine 1989;7(4):345–8.
[11] Christenson B, Bottiger M. Long-term follow-up study of rubella antibodies in naturally immune and vaccinated young adults. Vaccine 1994;12(1):41–5.
[12] Davidkin I, Peltola H, Leinikki P, Valle M. Duration of rubella immunity induced by two-dose measles, mumps and rubella (MMR) vaccination. A 15-year follow-up in Finland. Vaccine 2000;18(27):3106–12.
[13] Banatvala JE, Brown DW. Rubella. Lancet 2004;363(9415):1127–37.
[14] Christenson B, Bottiger M. Measles antibody: comparison of long-term vaccination titres, early vaccination titres and naturally acquired immunity to and booster effects on the measles virus. Vaccine 1994;12(2):129–33.
[15] Dai B, Chen ZH, Liu QC, Wu T, Guo CY, Wang XZ, et al. Duration of immunity following immunization with live measles vaccine: 15 years of observation in Zhejiang Province, China. Bull World Health Organ 1991;69(4):415–23.
[16] Davidkin I, Valle M. Vaccine-induced measles virus antibodies after two doses of combined measles, mumps and rubella vaccine: a 12-year follow-up in two cohorts. Vaccine 1998;16(20):2052–7.
[17] Krugman S. Further-attenuated measles vaccine: characteristics and use. Rev Infect Dis 1983;5(3):477–81.
[18] Lee MS, Chien LJ, Yueh YY, Lu CF. Measles seroepidemiology and decay rate of vaccine-induced measles IgG titers in Taiwan. Vaccine 2001;19(32):4644–51.
[19] Mossong J, O'Callaghan CJ, Ratnam S. Modelling antibody response to measles vaccine and subsequent waning of immunity in a low exposure population. Vaccine 2000;19(4–5):523–9.
[20] Mossong J, Putz L, Schneider F. Seroprevalence of measles, mumps and rubella antibodies in Luxembourg: results from a national cross-sectional study. Epidemiol Infect 2004;132(1):11–8.
[21] Ratnam S, West R, Gadag V, Williams B, Oates E. Immunity against measles in school-aged children: implications for measles revaccination strategies. Can J Public Health 1996;87(6):407–10.
[22] van den Hof S, Berbers GA, de Melker HE, Conyn-van Spaendonck MA. Sero-epidemiology of measles antibodies in the Netherlands, a cross-sectional study in a national sample and in communities with low vaccine coverage. Vaccine 1999;18(9–10):931–40.
[23] Dopatka HD, Giesendorf B. Single point quantification of antibody by ELISA without need of a reference curve. J Clin Lab Anal 1992;6(6):417–22.
[24] Mossong J, Muller CP. Estimation of the basic reproduction number of measles during an outbreak in a partially vaccinated population. Epidemiol Infect 2000;124(2):273–8.
[25] Kremer JR, Bouche FB, Schneider F, Muller CP. Re-exposure to wild-type virus stabilizes measles-specific antibody levels in late convalescent patients. J Clin Virol 2005.
[26] Cusi MG, Rossolini GM, Valensin PE, Cellesi C, Zanchi A. Serological evidence of reinfection among vaccinees during rubella outbreak. Lancet 1990;336(8722):1071.
[27] Cusi MG, Valensin PE, Cellesi C. Possibility of reinfection after immunisation with RA27/3 live attenuated rubella virus. Arch Virol 1993;129(1–4):337–40.
[28] O'Shea S, Best JM, Banatvala JE. Viremia, virus excretion, and antibody responses after challenge in volunteers with low levels of antibody to rubella virus. J Infect Dis 1983;148(4):639–47.
[29] Markowitz LE, Preblud SR, Orenstein WA, Rovira EZ, Adams NC, Hawkins CE, et al. Patterns of transmission in measles outbreaks in the United States. N Engl J Med 1989;320(2):75–81.
[30] Mathias RG, Meekison WG, Arcand TA, Schechter MT. The role of secondary vaccine failures in measles outbreaks. Am J Public Health 1989;79(4):475–8.
[31] Hirose M, Hidaka Y, Miyazaki C, Ueda K, Yoshikawa H. Five cases of measles secondary vaccine failure with confirmed seroconversion after live measles vaccination. Scand J Infect Dis 1997;29(2):187–90.

[32] Reyes MA, de Borrero MF, Roa J, Bergonzoli G, Saravia NG. Measles vaccine failure after documented seroconversion. Pediatr Infect Dis J 1987;6(9):848–51.
[33] Smith FR, Curran AS, Raciti KA, Black FL. Reported measles in persons immunologically primed by prior vaccination. J Pediatr 1982;101(3):391–3.
[34] Muller CP, Huiss S, Schneider F. Secondary immune responses in parents of children with recent measles. Lancet 1996;348(9038):1379–80.
[35] de Swart RL, Yuksel S, Osterhaus AD. Relative contributions of measles virus hemagglutinin- and fusion protein-specific serum antibodies to virus neutralization. J Virol 2005;79(17):11547–51.
[36] Cohen BJ, Parry RP, Doblas D, Samuel D, Warrener L, Andrews N, et al. Measles immunity testing: comparison of two measles IgG ELISAs with plaque reduction neutralisation assay. J Virol Methods 2005.
[37] Paunio M, Peltola H, Valle M, Davidkin I, Virtanen M, Heinonen OP. Twice vaccinated recipients are better protected against epidemic measles than are single dose recipients of measles containing vaccine. J Epidemiol Community Health 1999;53(3):173–8.
[38] Vitek CR, Aduddell M, Brinton MJ, Hoffman RE, Redd SC. Increased protections during a measles outbreak of children previously vaccinated with a second dose of measles-mumps-rubella vaccine. Pediatr Infect Dis J 1999;18(7):620–3.

VII. Stabilisation des anticorps spécifiques de la rougeole par réexposition au virus sauvage

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Résumé de l'article

Il est généralement accepté, qu'une infection naturelle par une souche sauvage de la rougeole induit des taux d'anticorps élevés, protégeant à vie contre la maladie. Cependant il a été observé, que les convalescents peuvent développer une réponse immunitaire secondaire, après un autre contact avec une souche sauvage. Le but de la présente étude est de déterminer l'effet à long terme d'une réexposition à une souche sauvage sur l'immunité humorale plusieurs années après la convalescence.

Deux sérums consécutifs furent prélevés auprès de 43 convalescents 1-6 ans avant ainsi que 3.7-4.8 après une réexposition prolongée et documentée à une souche sauvage du virus de la rougeole (groupe LC+). L'évolution des taux d'anticorps dans ce groupe fut comparée à celle d'un deuxième groupe de 43 convalescents du même âge et même sexe, mais sans réexposition documentée à une souche sauvage pendant la même période (LC-). De plus les anticorps anti-rougeole furent déterminés dans deux sérums consécutifs, prélevés auprès d'un groupe de 26 individus 1.3-1.7 et 3.8-4.1 ans après convalescence (groupe EC). Dans tous les sérums, les taux d'anticorps furent déterminés par ELISA (Enzygnost, Dade-Behring, Marburg, D).

Dans le groupe LC-, la moyenne géométrique (MG) des taux d'anticorps avait diminuée de 12.1% (intervalle de confiance (IC) : 3.2-20.3%, p=0.01) entre les deux prélèvements de sérum (intervalle de temps : 4.1-10.0 ans ; moyenne 6.3 ans). De même une diminution de la MG, bien que statistiquement peu significative, fut observé dans le groupe EC pendant une période de 2.6 ans (-6.5% ; IC: −13.3% to +0.1% ; p=0.08). Ces observations indiquent que les taux d'anticorps induits par les souches sauvages du virus diminuent après convalescence.

Nous avions précédemment montré que quatre participants du groupe LC+ avaient développé une réponse immunitaire secondaire après réexposition à une souche sauvage en 1996. Cette réponse immunitaire avait été identifiée par une augmentation significative des taux d'anticorps au contact de la souche sauvage. Nous montrons ici que plus de 4 ans après réexposition les

taux d'anticorps étaient toujours plus élevés qu'avant réexposition bien que la demi-vie des anticorps stimulés ait été relativement courte (3.5-5.0 ans). D'autre part la MG déterminée dans les sérums prélevés avant et après réexposition auprès des 39 autres participants du groupe LC+ (intervalle de temps : 5.1-11.1 ans ; moyenne 7.7 ans) était pratiquement identique. Ceci suggère que les taux d'anticorps ont été stabilisés au moins temporairement au fait de la réexposition à une souche sauvage, malgré l'absence d'une réponse immunitaire secondaire détectable.

Les résultats de cette étude montrent que les taux d'anticorps diminuent après convalescence, et peuvent être stabilisés par réexposition au virus sauvage. Cependant le déclin des taux d'anticorps après convalescence semble négligeable par rapport au taux initial induit par l'infection. Ainsi, même après l'absence prolongée de souche virale sauvage tous les convalescents resteraient protégés contre la maladie.

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Short communication

Re-exposure to wild-type virus stabilizes measles-specific antibody levels in late convalescent patients

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Abstract

Background: Infection with wild-type (wt) measles virus strains induces high antibody levels believed to provide life-long protection against disease.

Objectives: Humoral immunity was followed up in convalescent measles patients to assess the persistence of specific antibodies after measles disease in individuals without and with documented re-exposure to wt virus.

Study design: Paired sera were collected from 43 late convalescents (LC) before re-exposure and 3.7–4.8 years after re-exposure to at least one measles patient (LC+ group). Antibody persistence in this group was compared to paired sera from 43 age- and sex-matched controls without documented exposure to wt virus (LC− group). Paired sera were also obtained from 26 measles patients 1.3–1.7 and 3.8–4.1 years after they had recovered from measles to observe the waning of antibodies in early convalescents (EC group).

Results: Antibody levels decreased by 12.1% (CI: 3.2–20.3%, $p=0.01$) within 6.3 years in the LC− group of late convalescent measles patients. In contrast, in the LC+ group GMT of first and second sera were virtually identical, indicating that exposure to wt virus stabilizes antibody levels even in absence of a detectable secondary immune response. In a subset of late convalescents of group LC+ with a secondary immune response, antibody waning after re-exposure was as high as 15.6%/year (CI: 13.0–17.7%/year), corresponding to a half-life of 4.1 years (CI: 3.5–5.0 years), but antibodies were still higher than before re-exposure. In the EC group GMT decreased by 6.5% (95% CI: −13.3% to +0.1%) during 2.5 years but significance was low ($p=0.08$).

Conclusion: The maintenance of antibody levels in convalescent measles patients is at least partially dependant on recurrent exposure to circulating wt virus.

Keywords: Wild-type virus; Convalescent measles; Antibody level

1. Introduction

Reinforced vaccination schedules have dramatically decreased measles morbidity and mortality, and have effectively interrupted circulation of indigenous virus in most of the Americas and a number of other countries (CDC, 2004; Hanses et al., 2000; Muller et al., 2002). Before the introduction of vaccination, children developed measles during early childhood. Wild-type (wt) measles virus (MV) infection induces high titres of antibodies (Damien et al., 1998), which are found in >99% of individuals of unvaccinated birth cohorts (Mossong et al., 2004; van den Hof et al., 1999) and natural immunity is considered to be life-long (Panum, 1939). We have shown before that late convalescent patients with relatively low antibody levels may develop a secondary immune response (SIR), resulting in a significant antibody increase, after re-exposure to measles patients (Huiss et al., 1997; Muller et al., 1996). Here specific antibodies were followed up in late and early convalescent measles patients to assess the persistence of specific antibodies after measles disease in individuals without and with documented re-exposure to wt virus.

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2. Methods

2.1. Cohorts

A first group consisted of 26 early convalescent measles patients (EC). The patients were 1.3–24.9 years (median: 6.7 years) old when they had measles. Cases were serologically confirmed during a primary school-based outbreak in the North of Luxembourg during March–June 1996 (Hanses et al., 2000; Mossong and Muller, 2000). A second and third serum was obtained from every individual 1.3–1.7 and 3.8–4.1 years after measles (mean intervall: 2.5 years; range 2.4–2.6 years).

A second group consisted of 43 late convalescent household contacts (LC+), re-exposed to measles patients during the same outbreak in 1996 (Huiss et al., 1997). Most were mothers (83.7%) attending to their measles child. Participants were between 26.4 and 67.4 years of age (median age: 35.7 years) at the moment of re-exposure. Because of their age, these were considered as measles late convalescents, since vaccination against measles was very rare in these birth cohorts. A "pre-re-exposure" serum from these individuals, collected between 1990 and 1995, was selected from the serum bank of the National Public Health Laboratory (Luxembourg). A second and third serum was collected in 1996, 2–12 weeks (re-exposure) and in 2001–2002, 3.7–4.8 years (post-re-exposure) after measles re-exposure. The mean time intervall between pre- and post-re-exposure sera was 7.7 years (range: 5.1–11.1 years).

For each of the above LC+ individuals, an age- and sex-matched control person, without confirmed re-exposure to measles patients was identified in our serum bank, forming the LC− group ($n=43$). From these control individuals paired sera collected during 1990–2002, were also available (mean intervall: 6.3 years; range: 4.1–10.0). The study was approved by the local ethics committee and the responsible ministries. Informed consent was obtained from all participants.

3. Measles virus antibodies

Serum anti-MV IgG levels were assessed by enzyme immuno assay (EIA) (Enzygnost®, Dade-Behring, Marburg, Germany). All sera were tested together in a single large experiment and paired sera of the same individuals were tested in the same microtiter plate. Following the manufacturers instructions, sera were considered anti-MV IgG positive if the corrected absorbance was higher than 200 mOD. Antibody titres were calculated in milli-international units per millilitre (mIU/ml) using the alpha method (Dopatka and Giesendorf, 1992), and three-fold differences in titre were considered significant, according to the manufacturers guidelines. Statistical significance between geometric mean titres (GMT) was calculated using SigmaStat version 3.11 software (Systat software Inc., Erkrath, Germany) with two-tailed paired or unpaired Student's t-test after confirming normal distributions of individual titres (log mIU/ml), or by Wilcoxon Signed Rank test when individual titres had no normal distribution.

4. Results and discussion

All participants were seropositive for MV in all sera. In the 43 late convalescent donors with documented re-exposure to wt virus (LC+), the GMT was somewhat higher in the post-re-exposure compared to pre-re-exposure sera (Fig. 1), although this difference was not statistically significant ($p=0.2$). We have previously shown, that among these 43 LC+ participants, four developed a SIR associated with a significant increase (>five-fold) in specific IgG 2–12 weeks after re-exposure to measles patients (Huiss et al., 1997; Muller et al., 1996). All four individuals with SIR had relatively low pre-re-exposure antibody levels (<3000 mIU/ml) compared to those without SIR. Here we show that in these late convalescents with SIR, antibodies boosted by wt virus had returned to significantly lower levels 3.9 years post-re-exposure (Fig. 2). An annual antibody decay rate of 15.6% (95% confidence intervall (CI): 13.0%–17.7%), corresponding to an antibody half-life of 4.1 years (CI: 3.5–5.0 years), was estimated from the difference in GMT between "re-exposure" and "post-re-exposure" sera of the four participants with SIR. Moreover 3.9 years after re-exposure, antibody titres were still 2.2–7.5 times higher than before re-exposure. This suggests that wt virus induces a significantly prolonged antibody boost in comparison to revaccination, since most studies in vaccinees suggest that pre-boosting levels are reached within less than one year after revaccination (Davidkin and Valle, 1998; Deseda-Tous et al., 1978; Krugman, 1983). When the above

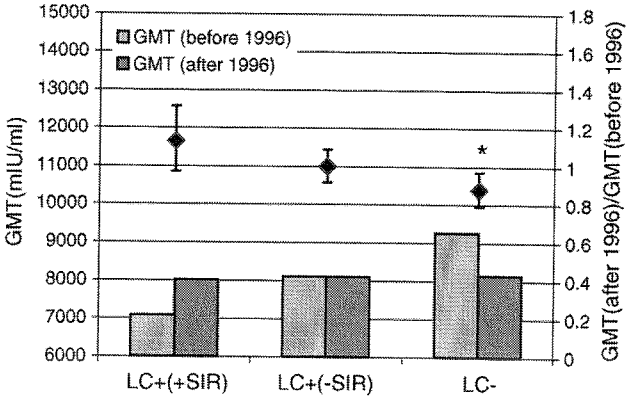

Fig. 1. GMTs of measles IgG in paired sera of age and sex-matched late convalescents with (LC+, $n=43$) and without (LC−, $n=43$) documented exposure to measles patients in 1996, with (+SIR) or without (−SIR) the four LC+ individuals with secondary immune response included. GMTs (mIU/ml) were calculated from the individual titres (mIU/ml) in pre- (before 1996) and post-re-exposure (2000–2001) sera of LC+, as well as in paired sera from LC− participants collected before and after 1996. The ratios of GMTs (±95% CI) of paired sera (after 1996/before 1996) are also shown (♦). Stars (*) indicate statistical significance by paired Student's t-test ($p<0.05$).

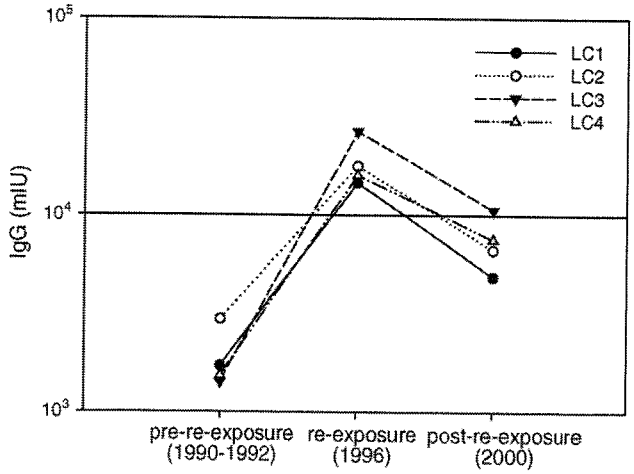

Fig. 2. Evolution of anti-MV IgG titres in four late convalescent individuals (LC1-4) with secondary immune response upon contact with a measles patient. Sera were obtained 4–6 years before, about 1 month ("re-exposure") and 3.9 years after re-exposure.

four individuals with detectable SIR were excluded from LC+ group GMT in pre- and post-re-exposure sera was virtually identical (Fig. 1). In contrast, GMT of the age- and sex-matched control group (LC−), without known exposure, decreased by 12.1% (CI: 3.2% to 20.3%; $p=0.01$), even though the interval between sera was somewhat shorter.

The longitudinal comparison of paired sera of the EC group showed that GMT also decreased by 6.5% (CI: −13.3% to +0.1%) within 2.5 years, but significance of this waning was low ($p=0.08$), probably because of the small number of participants and the short observation period. Moreover GMT (mIU/ml) was significantly higher (1.6-fold; $p<0.001$) in the sera of early than in those of late convalescents (Fig. 3), despite two early convalescents, with unknown vaccination status, who had low antibody

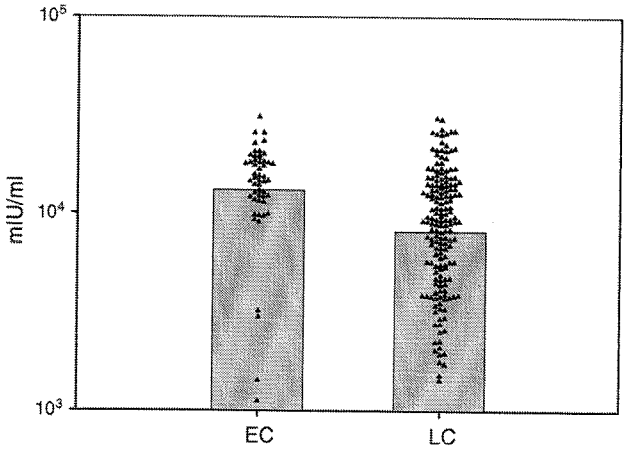

Fig. 3. Measles IgG levels in paired sera from 26 early convalescents (EC) 1.3–1.7 and 3.8–4.1 years after measles as well as paired sera collected between 1990–1995 and 1997–2003 from 86 late convalescents (LC) with (LC+, $n=43$) and those without (LC−, $n=43$) documented exposure to wt virus in 1996 were included. Triangles represent titres (mIU/ml) of individual donors and bars indicate the GMT (mIU/ml) of all sera for each group.

levels, perhaps due to vaccine-modified measles. The latter cross-sectional analysis thus also confirms, that antibodies naturally wane after wt infection.

Although measles was present in the neighbouring countries, only few unrelated measles cases were reported from Luxembourg after the last major outbreak in 1996 (Hanses et al., 2000). It can therefore be assumed that exposure to wt virus was rare during the study period, although it cannot be fully excluded, in particular since the role of subclinical infection in MV circulation is poorly understood. Nevertheless the household exposure to measles was much more intense than any fortuitous contact of the LC− group. Therefore, we can conclude that in the absence of a sizeable natural boost with wt virus, measles-specific antibodies seem to decay, similarly to the well-documented waning of antibodies in vaccinees. Furthermore we show here for the first time, that the maintenance of antibody levels in late convalescent measles patients is at least partially the result of recurrent contacts with wt virus, even if no measurable SIR suggests such a contact. Nevertheless, the absence of circulating wt virus most probably will not compromise life-long protection after measles disease, even if only the humoral response is considered. With an estimated half-life of about 35 years (assuming 2% waning/year), antibody waning is slow in comparison to the absolute antibody levels induced by wt infection: it would take more than four half-lives until the first late convalescents of our study would fall below titres that are thought to be protective (250 mIU/ml). Our results show also that wt boosts induce a prolonged increase of antibody levels and may therefore lead to a significant overestimation of antibody half-lives, especially in cross-sectional studies where SIRs remain unnoticed.

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References

CDC. Progress toward measles elimination — region of the Americas, 2002–2003. MMWR Morb Mortal Weekly Rep 2004;53(14):304–6.

Damien B, Huiss S, Schneider F, Muller CP. Estimated susceptibility to asymptomatic secondary immune response against measles in late convalescent and vaccinated persons. J Med Virol 1998;56(1):85–90.

Davidkin I, Valle M. Vaccine-induced measles virus antibodies after two doses of combined measles, mumps and rubella vaccine: a 12-year follow-up in two cohorts. Vaccine 1998;16(20):2052–7.

Deseda-Tous J, Cherry JD, Spencer MJ, Welliver RC, Boyer KM, Dudley JP, et al. Measles revaccination. Persistence and degree

of antibody titer by type of immune response. Am J Dis Child 1978;132(3):287–90.

Dopatka HD, Giesendorf B. Single point quantification of antibody by ELISA without need of a reference curve. J Clin Lab Anal 1992;6(6):417–22.

Hanses F, van Binnendijk R, Ammerlaan W, Truong AT, de Rond L, Schneider F, et al. Genetic variability of measles viruses circulating in the Benelux. Arch Virol 2000;145(3):541–51.

Huiss S, Damien B, Schneider F, Muller CP. Characteristics of asymptomatic secondary immune responses to measles virus in late convalescent donors. Clin Exp Immunol 1997;109(3):416–20.

Krugman S. Further-attenuated measles vaccine: characteristics and use. Rev Infect Dis 1983;5(3):477–81.

Mossong J, Muller CP. Estimation of the basic reproduction number of measles during an outbreak in a partially vaccinated population. Epidemiol Infect 2000;124(2):273–8.

Mossong J, Putz L, Schneider F. Seroprevalence of measles, mumps and rubella antibodies in Luxembourg: results from a national cross-sectional study. Epidemiol Infect 2004;132(1):11–8.

Muller CP, Huiss S, Schneider F. Secondary immune responses in parents of children with recent measles. Lancet 1996;348(9038):1379–80.

Muller CP, Mulders MN. Molecular epidemiology of measles virus. In: Leitner T, editor. The molecular epidemiology of human viruses. Boston: Kluwer Academic Publishers; 2002. p. 237–72.

Panum P. Observations made during the epidemic of measles on the Faroe Islands in the year 1849. 1939.

van den Hof S, Berbers GA, de Melker HE, Conyn-van Spaendonck MA. Sero-epidemiology of measles antibodies in the Netherlands, a cross-sectional study in a national sample and in communities with low vaccine coverage. Vaccine 1999;18(9/10):931–40.

VIII. Evaluation d'un test ELISA commercialisé pour la détection des IgG spécifiques de la rougeole dans du fluide buccal de personnes vaccinées

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Résumé de l'article

Les études séro-épidémiolgiques sont importantes pour analyser l'immunité d'une population contres différents pathogènes. Alors que les taux d'anticorps sont en général mesurés dans le sérum, la détection d'IgG dans le fluide buccal a été évaluée pour plusieurs virus. Le prélèvement de fluide buccal a un grand nombre d'avantages par rapport au prélèvement sanguin. Cette étude a évalué la performance d'un test ELISA commercialisé pour détecter les IgG spécifiques de la rougeole dans le fluide buccal.

Des sérums et des fluides buccaux ont été prélevés en parallèle chez 283 jeunes adultes (âge: 18.1-21.9 ans) dont la majorité (87.3%) avait reçu une ou deux doses de vaccin contre la rougeole pendant leur enfance ou leur adolescence. Les IgG spécifiques de la rougeole ont été mesurés par la méthode Enzygnost (Dade-Behring, Marburg), et les anticorps dans le fluide buccal ont été quantifiés par une méthode développée spécialement pour ce type d'échantillon (Microimmune Ltd., Middlesex). La séroprévalence obtenue dans le sérum (89.0%, intervalle de confiance (IC): 84.8-92.4%) était considérablement supérieure à celle déterminée dans les fluides buccaux (81.6%, IC: 76.6%-86%). En considérant les résultats obtenus dans le sérum comme référence standard, il a été observé que la sensibilité de détection était plus faible dans le fluide buccal lorsque les taux d'anticorps dans le sérum étaient bas (<80% pour des taux d'anticorps dans le sérum inférieurs à 1000 milli unités internationales). Puisque les taux d'anticorps induits par vaccination sont en général relativement bas, les taux de séroprévalence peuvent donc être considérablement sous-estimés en utilisant la présente méthode.

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Evaluation of Commercial Assay Detecting Specific Immunoglobulin G in Oral Fluid for Determining Measles Immunity in Vaccinees

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A commercial assay for detection of measles immunoglobulin G (IgG) in oral fluid was evaluated in a highly vaccinated cohort using serum IgG as gold standard. In contrast to previous studies from cohorts protected by natural immunity, antibody prevalence was significantly underestimated (−7.4%; confidence interval: −1.5 to −13.2%; $P = 0.01$) due to a reduced sensitivity when antibody levels were low.

Vaccination strategies to control and eliminate measles have been widely monitored by seroepidemiological studies. The prevalence of immunoglobulin G (IgG) antibodies in the blood is considered a solid correlate of population immunity. A major drawback of serum IgG is the need to draw blood with all its risks and ethical implications. Therefore, the detection of IgG in oral fluid, which can be collected with relative ease, has been promoted as a noninvasive alternative for seroprevalence studies (6). Specific IgG of a number of viruses has been found in oral fluid (3, 4, 7, 8). Nigatu et al. have developed an antibody capture enzyme immunoassay (EIA) to detect measles IgG in oral fluid, which was recently commercialized (5). Estimates of antibody prevalence in two cohorts from Ethiopia with low vaccination coverage did not significantly differ when IgG in oral fluid and serum were compared (5, 6). The aim of the present study was to evaluate IgG detection in oral fluid in a highly vaccinated central European community.

Paired serum and oral fluid samples were collected from 283 school-children (age range: 18.1 to 21.9; mean age: 19.6; male: 20.1%) attending three different secondary schools in Luxembourg. Most (87.3%) of them had received one or two doses of measles vaccine during early childhood and/or adolescence. The others were not vaccinated (6.0%) or had unreliable vaccination records (6.7%). Seventeen individuals with high serum antibody titers were not vaccinated and are considered convalescent measles patients. Informed consent was obtained from all participants or their guardians. The study was approved by the local ethics committee and the responsible ministries.

Peripheral blood was obtained by venipuncture and incubated for 1 h at 37°C. Serum was collected after centrifugation for 25 min at 1,700 × *g*, divided into aliquots, and stored at −20°C until testing. Anti-measles virus IgG levels in the serum were assessed by the World Health Organization-recommended EIA (Enzygnost; Dade-Behring, Marburg) with a validated sensitivity and specificity of 99.6 and 100%. Antibody status (positive >0.2 optical densities [OD]; negative <0.1 OD; or equivocal 0.1 to 0.2 OD) and concentrations (mIU/ml) were determined using the alpha method (2) according to the manufacturer's protocol.

Oral fluid was collected as described elsewhere (7) by moving a cylindrical sponge (Oracol; Malvern Medical Developments, Worcester, United Kingdom) around the gums for about 1 min and kept at 4°C until processing. Within a maximum of 4 h postcollection oral fluid was extracted from the sponge by centrifugation (700 × *g*, 5 min) and immediately stored undiluted at −20°C. Antibody levels were determined using a commercial EIA recommended and optimized for oral fluid (Microimmune Ltd., Middlesex) following strictly the manufacturer's instructions (positive, >1.25× the mean OD of three negative controls [ODnc]); negative, <1.1× ODnc; equivocal, between 1.1 to 1.25× ODnc). Briefly total IgG from undiluted oral fluid was captured in anti-human IgG coated microtiter wells. Recombinant measles nucleoprotein (rMVN), a monoclonal antibody to rMVN conjugated to horseradish peroxidase and the substrate (tetramethylbenzidine) were sequentially added to reveal the presence of measles-specific antibodies. Sensitivity, specificity, and positive and negative predictive values of the oral fluid assay were determined by comparison to serum IgG as described by Nokes et al. (7) using the Enzygnost test as a gold standard. Exact binomial confidence intervals of proportions and differences between proportions (z-test) were determined using SigmaStat version 3.0.1a software (Systat software Inc., Point Richmond).

Measles specific IgG was measured in 283 paired oral fluid and serum samples. Numbers of individuals with positive, negative, or equivocal absorbance values in the two assays are shown in Table 1. When equivocal absorbances in either of the assays were excluded, prevalence estimates obtained from serum (89.0%, confidence interval [CI]: 84.8 to 92.4%) were significantly higher than those for oral fluid (81.6%, CI: 76.6 to 86.0%). Higher values but similar differences were obtained when equivocal values were included as positives (serum, 95.4%, CI: 92.3 to 97.5%; oral fluid, 88.3%, CI: 84.0 to 91.8%). A concordant antibody status (positive, negative, equivocal) was found in 227 individuals (80.2%). When individuals with equivocal absorbances in either test were excluded, a concordance of 90.0% was observed.

When the serum assay was taken as a gold standard and individuals with equivocal results in either test (12.0%) were

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TABLE 1. Numbers of individuals by measles-specific antibody status (positive, negative, equivocal) in serum and oral fluid

Oral fluid	Serum			Total
	Positive *n* (%)	Negative *n* (%)	Equivocal *n* (%)	
Positive *n* (%)	217 (76.7)	2 (0.7)	12 (4.2)	231 (81.7)
Negative *n* (%)	23 (8.1)	7 (2.5)	3 (1.1)	33 (11.6)
Equivocal *n* (%)	12 (4.2)	4 (1.4)	3 (1.1)	19 (6.7)
Total	252 (89.0)	13 (4.6)	18 (6.4)	283 (100)

excluded, sensitivity and positive predictive values of the oral fluid assay were 90.4% (CI: 86.0 to 93.8%) and 99.1% (CI: 96.7 to 99.9%), respectively. The specificity (77.8%; CI: 40.0 to 97.2%) as well as negative predictive values were lower (23.3%; CI: 9.9 to 42.3%), but the number of seronegative individuals (n = 13) was limited.

Figure 1 shows that the oral fluid assay misses many of the individuals with low specific serum IgG. Sensitivity was significantly lower in our cohort of central European vaccinees than those determined by Nokes et al. (6) in a cohort from rural Ethiopia (97%). Very low and low vaccination coverage rates (16.6 to 54.8%) were reported in that study from rural and urban communities in Ethiopia. Furthermore, about half of the individuals were >20 years old and unlikely to have been vaccinated. The rural cohort may have consisted of 85% and more of late convalescent measles patients. Natural infection normally induces much higher, by some accounts 5 to 10 times

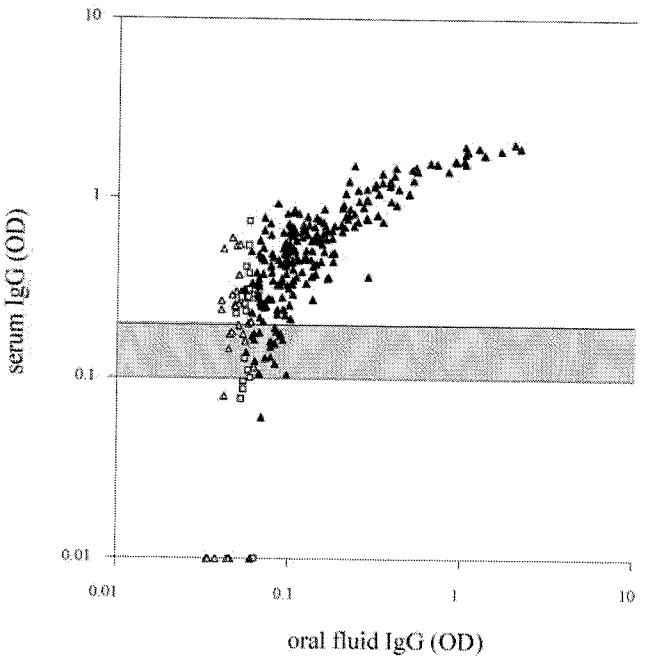

FIG. 1. Comparison of measles-specific IgG (OD values) in serum and oral fluid of 283 adolescents. Horizontal lines indicate cutoff values of the serum assay (negative, <0.1 OD; positive, >0.2 OD; greyzone, 0.1 to 0.2 OD). IgG-positive (▲), -negative (△), and -equivocal (□) oral fluid samples. In contrast to the serum assay, greyzone intervals of the oral fluid assay are defined with respect to negative controls on the same test plate and not in absolute OD values.

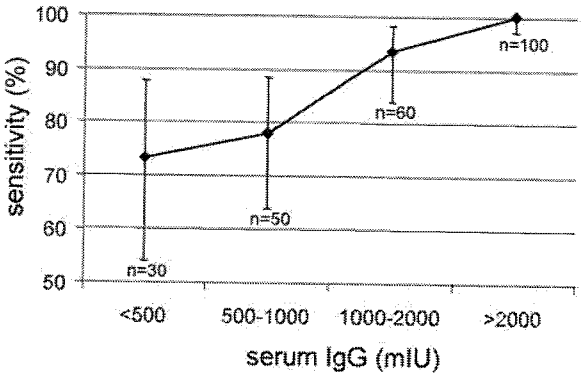

FIG. 2. Sensitivity and CI of the oral fluid test for different levels of serum IgG (mIU). Number of individuals per category are given.

higher, antibodies than vaccination (1), similar to rubella (9). Thus, the discrepancies in sensitivity may be fully explained by the differences in antibody levels in individuals with wild-type virus or vaccine-induced immunity. To compensate for low antibody concentrations, the oral fluid EIA is based on total IgG capture technology (5). In contrast, the serum EIA binds measles-specific antibody only. This may partially account for the difference in sensitivity between both methods. Figure 2 shows that the sensitivity of the oral fluid method increases with higher serum antibody levels. The lowest sensitivity (73.3%, CI: 54.1 to 87.7%) was found among individuals with antibody titers below 500 mIU (0.28 OD) and gradually increased to 100% (CI: 97.1 to 100%) when serum antibodies reached >2,000 mIU (0.65 OD). As a consequence seroprevalence in a highly vaccinated community is underestimated by oral fluid IgG detection due to an insufficient sensitivity of the assay in the low titer range. However, the oral fluid test may be useful to identify individuals with no or low serum antibodies for revaccination. In this case a high specificity must be warranted to avoid that seronegative individuals would be missed. In the present cohort, 2 of 13 seronegative individuals gave weak but false-positive results in the oral fluid assay. Although only weakly positive, these false positive donors could only be excluded at the expense of higher number of false negatives. For instance, if the positive threshold is raised from 1.25× to 1.5× ODnc, the number of greyzone individuals would increase from 19 to 59 in this study. A careful reevaluation of the positive/negative cutoffs even in a cohort of vaccinees including sufficient numbers of seronegative donors may not necessarily be the solution. In general, overvaccination would be preferred to undervaccination.

Our results show that characteristics, in particular the sensitivity of measles antibody assays, largely depend on the vaccination status of the cohort and should be evaluated not only in late convalescents but also in vaccinees.

We thank all participants for donating blood and oral fluid. We also thank Ulla Muller, Evguenia Pasthukova, and Stephanie Willième for administrative and technical support. We also acknowledge the support of the Centre de Recherche Public-Santé, the Ministère de la Recherche, the Ministère de la Santé, and the participating schools.

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REFERENCES

1. **Damien, B., S. Huiss, F. Schneider, and C. P. Muller.** 1998. Estimated susceptibility to asymptomatic secondary immune response against measles in late convalescent and vaccinated persons. J. Med. Virol. **56**:85–90.
2. **Dopatka, H. D., and B. Giesendorf.** 1992. Single point quantification of antibody by ELISA without need of a reference curve. J. Clin. Lab. Anal. **6**:417–422.
3. **Granade, T. C., S. K. Phillips, B. Parekh, P. Gomez, W. Kitson-Piggott, H. Oleander, B. Mahabir, W. Charles, and S. Lee-Thomas.** 1998. Detection of antibodies to human immunodeficiency virus type 1 in oral fluids: a large-scale evaluation of immunoassay performance. Clin. Diagn. Lab. Immunol. **5**:171–175.
4. **Moe, C. L., A. Sair, L. Lindesmith, M. K. Estes, and L. A. Jaykus.** 2004. Diagnosis of Norwalk virus infection by indirect enzyme immunoassay detection of salivary antibodies to recombinant Norwalk virus antigen. Clin. Diagn. Lab. Immunol. **11**:1028–1034.
5. **Nigatu, W., D. J. Nokes, F. Enquselassie, D. W. Brown, B. J. Cohen, A. J. Vyse, and F. T. Cutts.** 1999. Detection of measles specific IgG in oral fluid using an FITC/anti-FITC IgG capture enzyme linked immunosorbent assay (GACELISA). J. Virol. Methods **83**:135–144.
6. **Nokes, D. J., F. Enquselassie, W. Nigatu, A. J. Vyse, B. J. Cohen, D. W. Brown, and F. T. Cutts.** 2001. Has oral fluid the potential to replace serum for the evaluation of population immunity levels? A study of measles, rubella and hepatitis B in rural Ethiopia. Bull. W. H. O. **79**:588–595.
7. **Nokes, D. J., W. Nigatu, A. Abebe, T. Messele, A. Dejene, F. Enquselassie, A. Vyse, D. Brown, and F. T. Cutts.** 1998. A comparison of oral fluid and serum for the detection of rubella-specific antibodies in a community study in Addis Ababa, Ethiopia. Trop. Med. Int. Health **3**:258–267.
8. **Sheppard, C., B. Cohen, N. Andrews, and H. Surridge.** 2001. Development and evaluation of an antibody capture ELISA for detection of IgG to Epstein-Barr virus in oral fluid samples. J. Virol. Methods **93**:157–166.
9. **Vyse, A. J., B. J. Cohen, and M. E. Ramsay.** 2001. A comparison of oral fluid collection devices for use in the surveillance of virus diseases in children. Public Health **115**:201–207.

IX. Epidémiologie moléculaire du virus de la Rougeole en Asie

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Résumé de l'article

Contrairement à ce qui fut fait sur d'autres continents, un nombre très limité de souches sauvages de la rougeole a été génotypé en Asie avant l'an 2000. Par conséquent les premières études d'épidémiologie moléculaire menées plus récemment en Asie ont identifié six nouveaux génotypes du virus de la rougeole: D8, D9, H1, H2, G2, G3. Il est important de connaître les génotypes endémiques des différentes régions pour prouver une interruption de la circulation par épidémiologie moléculaire. L'article ci-présent fournit une vue d'ensemble sur toutes les publications qui ont annoncé la circulation des différents génotypes du virus de la rougeole en Asie avant l'an 2003.

Le Japon est le seul pays du continent asiatique dans lequel un nombre significatif de souches sauvages a été génotypé depuis les années 1980. Alors que des souches appartenant au génotype C1 ont été identifiées seulement dans les années 80s, les génotypes D5 et D3 ont été détectés pendant les années 1990, avant l'apparition du génotype H1 en 2000. La présence successive, mais prolongée de différents génotypes, suggère que la circulation du virus de la rougeole a pu être interrompue à plusieurs reprises, avant de recirculer après importation.

Les souches sauvages qui circulaient dans différentes provinces chinoises au début des années 1990 ont toutes été attribuées au clade H. L'importante variabilité importante entre les différentes souches indiquait que la circulation du virus était endémique à ce moment là. Les premières souches analysées au Vietnam appartenaient aussi au clade H. A cause de la grande distance génétique par rapport aux virus chinois, le clade H a été subdivisé en deux génotypes H1 (Chine) et H2 (Vietnam). Le génotype H1 a aussi été identifié lors d'une épidémie en Corée du Sud. Cependant la diversité entre différentes souches était très limitée, ce qui suggère que la circulation de ce virus a résulté d'une importation, probablement en provenance de la Chine.

Un nouveau génotype (D8) a été identifié avec les premières souches népalaises caractérisées en 1999. D'autres études menées dans le subcontinent indien ont indiqué que dans cette région, les génotypes D8 et D4 circulent en parallèle.
Deux nouveaux génotypes, G2 et D9 ont été identifiés en Indonésie en 1999.
Lors d'une épidémie en Australie, dans un camp de réfugiés provenant du Timor oriental, un autre génotype inconnu (G3) a été détecté pour la première fois. Une première étude menée en Papouasie Nouvelle-Guinée a révélé que D3 est le génotype indigène de cette région.
En Russie trois génotypes différents A, D4 et D6 ont été identifiés, malgré la caractérisation de quelques souches seulement. Plusieurs souches appartenant au génotype A ont aussi été détectées en Chine et en Inde. Cependant la proximité génétique par rapport aux souches vaccinales indique que ces résultats ne démontrent pas nécessairement une circulation de virus sauvages du génotype A dans ces pays.
Ainsi les premières études d'épidémiologie moléculaire menées dans différents pays asiatiques ont démontré qu'une caractérisation régulière des souches sauvages circulant dans différentes régions est importante pour identifier tous les génotypes circulant au niveau global. Une circulation endémique du virus de la rougeole a été mise en évidence dans de nombreux pays asiatiques. Il sera important de caractériser les souches détectées après la mise en place de programmes de vaccination renforcés pour prouver l'interruption de la circulation des génotypes indigènes.

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Molecular epidemiology of measles in Asia

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ABSTRACT

Routine vaccination has considerably decreased global measles incidence and mortality, although less dramatically in Asia and many other developing countries. Molecular epidemiology is considered a powerful tool to enhance measles control and elimination. Based on sequence variability mainly of the H and N gene, measles virus strains are classified in 22 genotypes. Sequence information from the Asian continent was very limited until recently, and is still missing from a number of countries. During the past five years six new genotypes were found in Asia: D8 (Nepal), D9 (Indonesia), G2 (Indonesia, Malaysia), G3 (East Timor), H1 (China), H2 (Vietnam). High strain diversity in a number of countries like India, Nepal, China, Vietnam, Indonesia and Papua New Guinea suggests continued endemic transmission of the putative indigenous strains. Several rounds of genotype displacement (C1, D3, D5) reported since the early eighties from Japan reflect progress towards measles elimination. Some genotypes circulate in a more or less confined geographical region (e.g. H1, H2 in China and Vietnam) whereas others are found on most continents (e.g. D4, D6). The recurrent isolation of clade A strains, virtually identical to vaccine strains, in different Asian countries is conspicuous. Intensified control and vaccination efforts are required to move Asia away from endemic transmission.

1. INTRODUCTION

Measles is the most contagious airborne disease and causes high morbidity in particular in Asia and Africa. Immune-suppression and concurrent infections are the mean cause of often-fatal complications sustained by young children in many developing countries. Before vaccination, virtually every child had measles and was protected afterwards for life. Global measles incidence and mortality decreased dramatically after the introduction of routine vaccination. A single dose of the live-attenuated vaccine given at 9-15 months of age provides long-lasting protection [1]. However, to interrupt virus transmission 95% of a population must be immune [2]. Because of the difficulties to reach these levels of protection with a single dose routine immunisation, most countries have adopted a two-dose strategy or implemented mass vaccination campaigns. These reinforced vaccination schedules interrupted circulation of indigenous measles viruses in the US [3] and other countries [4,5]. However, even in highly vaccinated populations outbreaks continue to occur when the virus is reintroduced [4,6,7]. In countries with lower vaccination coverage the disease remains endemic. Most of the 40 million annual cases and an estimated 900,000 deaths occur in developing countries [8]. The success of enhanced vaccination and surveillance have encouraged the World Health Organization (WHO) to set-up a program for global measles control [9,10]. Molecular epidemiology of measles virus has proven to be a powerful tool to monitor virus circulation and interruption of measles transmission, a major step towards measles control and elimination [11].

2. THE MEASLES VIRUS

Measles virus (MV) is the prototype of the

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morbilliviruses, a genus belonging to the family of *Paramyxoviridae*. Humans are the only host. Some animal viruses including those more recently found in marine mammals belong to the same genus [12-15]. The human metapneumovirus [16] and the emerging zoonotic Hendra virus [17] and Nipah virus [18] are also members of the *Paramyxoviridae* family.

The monotypic MV has a linear, single stranded, non-segmented RNA of negative sense with about 15,900 ribonucleotides, spanning six open reading frames, encoding 8 proteins: the nucleocapsid (N), the phospho protein (P), the V and the C protein, the matrix protein (M), the fusion protein (F), the hemagglutinin protein (H) and the large protein or polymerase (L). As most RNA viruses, the MV uses its own RNA dependant RNA Polymerase, which lacks proof-reading activity. In vitro the virus' mutation rate has been estimated at about 9×10^{-5} per base per replication, and the genomic mutation rate at 1.43 per replication round [19]; in vivo estimates of mutation rates of $4 - 5 \times 10^{-4}$ per site per year were based on the genetic variability of two genotypes C2 and D3 [20]. The majority of mutations occur at the third base of a codon and are silent. Non-synonymous mutations may, however, influence virus ability to resist neutralisation by monoclonal antibodies [21,22] or even serum of vaccinees, at least in vitro [23].

3. PHYLOGENETIC ANALYSIS AND GENOTYPES

A comprehensive list of primers used for sequencing of measles virus genes is displayed on the website of the WHO Collaborating Center for Measles, Luxembourg (www.etat.lu/LNS). According to WHO recommendations [24,25], 450 nt of the C-terminus of the N-gene (the "hypervariable" region, HVR) is the minimal sequence required for genotyping (nucleotide pos. 1254 – 1709; numbering according to [26]). This part of measles genome shows the highest diversity (Fig 1). The full-length sequence of the H gene (pos. 7295 – 9148; numbering according to [26]) should also be obtained of every new reference strain, any suspected new genotype and -in fact- from a representative virus of each outbreak [24,25]. Phylogenetic trees are generated from multiple aligned sequences using standard programs such as ClustalX [27] and MEGA (version 2.1, [28,29]) or PHYLIP, a package of phylogeny programs [30]. These programs use subroutines such as the neighbor joining method [31], the unweighted pair group method with arithmetic mean (UPGMA) method [32] the maximum-likelihood method [33], the parsimony method [34]. Bootstrap analysis are performed as a statistical evaluation of evolutionary trees.

When the importance of molecular epidemiology for measles control was recognized, the WHO adopted a unique and unifying nomenclature for MV strains [24,25,35]. For instance, MVi/Janakpur.NEP/2.99/2 [36], designates the second MV isolate obtained in the city of Janakpur in Nepal the 2nd week in 1999. After sequencing the complete H and N genes the sequence was assigned to the genotype D8.

Eight clades (A – H) containing 22 different genotypes (A, B1-B3, C1, C2, D1-D9, E, F, G1-G3, H1 and H2; Fig. 2) have been defined [11]. The consensus is that genotypes should differ by at least 2.5% in nucleotides of the C-terminus of the N gene and 2.0% in the complete H gene [24,25].

Each genotype has a reference strain, which is normally the first isolate (Fig. 2). Reference strains are available from the WHO measles strain bank at the Centers of Disease Control and Prevention (CDC), Atlanta and the Central Public Health Laboratory, London. Our laboratory maintains a large collection of African and some Asian viruses (www.etat.lu/LNS).

4. GEOGRAPHIC DISTRIBUTION OF GENOTYPES

Most genotypes show a more or less distinct geographic distribution, others have been found throughout the world.

The reference strain of clade A (Edmonston wild-type strain, ATCC VR-24; Genbank AF266288) represents the earliest (1954) MV isolate [37]. Several vaccine strains (e.g. Schwarz, Moraten, Edmonston-Zagreb) have been obtained by extensively passaging the wild-type on different cell types. All other vaccine strains also belong to clade A despite their pretended distinct origins (e.g., Leningrad, Shanghai, Tanabe, AIK-C). During the past decades, clade A viruses have been isolated sporadically in most parts of the world including Asia. The high sequence homology or identity with vaccine strains, both over time and space warrants a discussion about the origin of these viruses (see Section 10).

Clade B consists of three genotypes: B1, B2 and B3 [22,38]. Only a few viruses of genotype B1 (Cameroon 1983) and B2 (Gabon 1984) were ever reported [39]; both genotypes are considered to be extinct [40]. B3 is

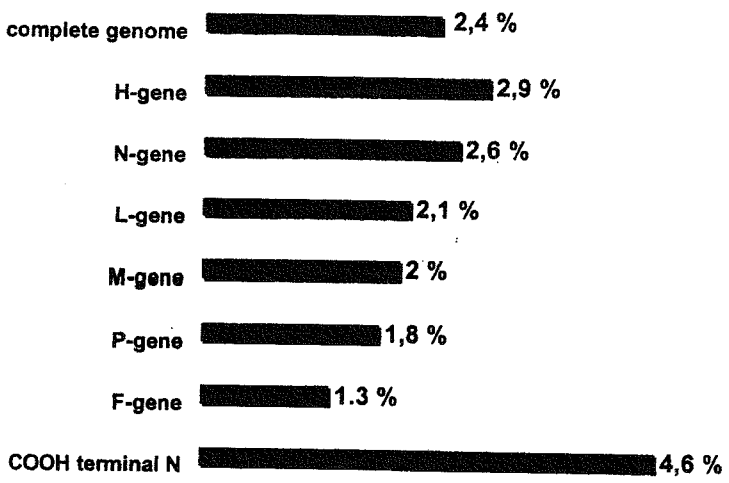

Figure 1: Sequence divergence between each MV gene of a D3 field isolate from Japan and the Edmonston wt strain [81].

the predominant genotype in Sub-Saharan Africa [22,38,41,42]. Two B3 viruses have also recently been found in Germany [43].

Clade C strains have been reported from all continents over the past 30 years. Genotype C2 was predominant mainly in Europe [4,20,44,45] and C1 is probably inactive.

With 9 different genotypes, clade D is the most heterogeneous; D1, formerly detected in Australia and the UK is considered inactive. The other clade D genotypes continue to be isolated in many parts of the world. D3, D4, D5 and D9 strains were mainly found in Australia, Asia and Southern Africa. D6 was the predominant European variant [3,4,45] but has been found during the recent outbreak that swept across Latin America as well [5,46-50].

Only a very limited number of clade E and F strains have been detected in Europe, the US and Canada [51-54]. These genotypes have not been seen since 1987 or the mid 1960's respectively and both lineages are considered to be extinct.

Viruses of clade G were originally found in the US during the early 1980s. Before the discovery of G2 and G3 strains in Indonesia and East Timor this clade was also considered to be inactive (see Section on Indonesia; [36,55-58])

Clade H predominates in South East Asia and has only sporadically been found outside of this region [59-62].

Intensified molecular characterization of measles isolates has revealed an increasing number of new genotypes during the past five years. Most of these previously unknown genotypes were found in Asia, highlighting the importance of molecular surveillance in these regions. In the following, the available sequence information of MV strains will be reviewed for the different regions in Asia.

5. JAPAN

The first MV isolates from Japan date back to 1971, allowing the phenotypic and genotypic characterisation of early wild type (wt) viruses [63]. Sakata et al. [64] identified three groups of wt viruses among isolates from Osaka, Tokyo, Nagoya and Akita between 1983 and 1990. Electrophoretic mobility of the H protein revealed a S (small) type with a molecular weight of 78 kDa, a M (medium) type and a L (large) type with a molecular weight of 80 and 82 kDa, respectively. The size difference was partially attributed to an additional potential glycosilation site in position aa416 of the M and the L type. Interestingly, the prevalence of the three types changed over the years. The S type dominated over the L type among the 1983/84 isolates from Osaka and Tokyo. During the late eighties only viruses of type M and L were found. Although limited, this data were a first indication that different lineages of MV were circulating concomitantly at the time in Japan and that viruses circulating in 1990 were different from those in

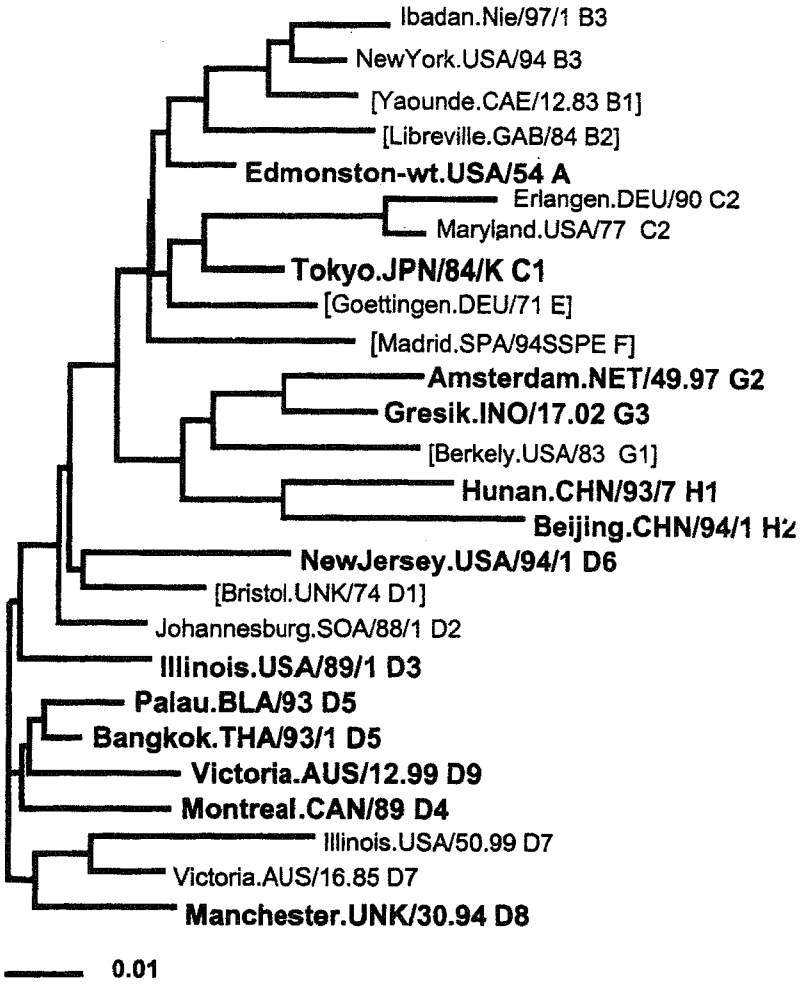

Figure 2: Phylogenetic tree based on the C-terminal region of the N-gene of the measles reference viruses used for genotype classification. ClustalX has been used to calculate the tree. Genotypes considered to be extinct are shown in brackets. Genotypes circulating in Asia are in bold.

the early eighties [64]. The genotype of these viruses has not been reported and their sequences were not submitted to a database.

In 1995, partial H- and N-sequences of Japanese strains collected between 1984 and 1991 became available [65]. These were phylogenetically classified into 3 groups initially referred to as A, B and C. According to the current WHO nomenclature these group A viruses correspond to genotype C1, group B strains to D3 and group C strains to D5. The genotype C1 was common before 1985, genotype D3 dominated during the late eighties and thereafter most of the isolates belonged to genotype D5. Thus, sequencing data confirmed the predominant and partially overlapping circulation of distinct wt strains during the eighties and nineties. In agreement with the observations by Sakata et al. [64] but in contrast to the later D3 and D5 viruses, the early C1 missed one additional (potential) glycosilation site in comparison to the later D3 and D5 viruses.

In another study, which included some of the above

strains, partial genes sequences of the phosphoprotein (P), matrix protein (M), fusion protein (F), large protein (L) as well as N and H protein of wt strains from Kanto district (1984-1994) were phylogenetically analysed [63]. The isolates from 1984 clustered within genotype C1; D3 strains were isolated between 1984 and 1989; all strains isolated between 1990 and 1994 belonged to genotype D5. The individual phylogenetic trees of each gene fragment showed similar topology and no evidence of recombination between lineages.

Interestingly, an isolate obtained in 1993 from a Japanese patient with subacute sclerosing panencephalitis (SSPE) and a history of measles in 1980 clustered with C1 strains isolated in 1984. Thus the SSPE isolate was most closely related to the strains that circulated during the early eighties in the country. Also a wt isolate from 1971 (Nagatha strain) was closely related to the C1 strains from 1984, which indicates that C1 may have been the predominant genotype until the early eighties [63]. This suggests that C1 may have been the indigenous genotype before large-scale vaccination was implemented in Japan, although some SSPE isolates and two wt strains of genotype C1 were also found in Europe (Spain 1978/1979) between the early 1970's and the mid-1980's. Unfortunately, the circumstances of the latter cases have not been reported [51].

During the Sapporo outbreak in 1995/1996 all viruses were classified as D5; in Tokio 1994-1997, almost 90% were D5 and only 10% were D3 viruses; two years later this ratio was reversed with more than 90% D3 viruses and <10% D5 isolates [66]. A large number of wt strains isolated between 1997-2001 in Osaka confirmed the co-circulation of genotypes D3 and D5, and their change in prevalence [67].

These phylogenetic data suggest a gradual genotype shift from the putative indigenous C1 viruses to D3 viruses in the eighties, displaced by D5 viruses in the early nineties before circulating again in the late nineties. D3 and D5 strains have also been isolated in other countries. The resurgence of measles in the US in 1989-1992 was caused by a single variant of D3 virus (0.5% diversity) distinct from the putative indigenous virus isolated last in 1983 [3]. D3 viruses were also found in the UK in 1988 [51]. As D3 viruses were detected already in 1984 [63] in Japan, phylogenetic analysis would be compatible with a Japanese origin of the above UK and US viruses. However, other early (indigenous) reservoirs of D3 viruses cannot be ruled out. D3 strains have also been found later in Taiwan (1994; [36]), in Papua New Guinea (1997 - 1999; [68]), and as an import from the Philippines again in the US (1996 and 2000; [7,69]). Whereas the strains from Taiwan and supposedly the Philippines were closely related to the early Japanese D3 isolates, the strains from Papua New Guinea form a distinct cluster within genotype D3 (Fig. 3). D5 strains have also been found in Thailand (1993) and Palau (1993) [3].

After the 1989-92 epidemic came to assend in the US sporadic cases were caused by viruses imported from throughout the world. In 1994 and 1996, D5 viruses were traced back to Japan [3,69,70] further confirming that this genotype displaced D3 in Japan. Between 1997 and 2001 Japan was again the source of infection for 6 of 10 sporadic cases and 2 minor outbreaks in the US [7]. All imported isolates were D5, with the exception of one D4 virus (2000). The latter finding was surprising since D5 was later partially displaced by D3 viruses and D4 has never been directly found in Japan. Similarly, several H1 strains have been isolated in Japan in 2001-2002 [71]. This genotype is indigenous in China and Korea and has never been isolated in Japan before. Sporadic cases caused by imported viruses that disappear rapidly is the typical epidemiological situation in a highly protected population where the dearth of susceptibles does not permit long chains of virus transmission.

6. CHINA, VIETNAM, KOREA

A first study from this part of the world reported the complete H and N nucleotide sequence of strains isolated in 1993/94 in 3 contiguous provinces of northeast (Shandong, Hebei and Beijing) and one province in southeast China (Hunan) [59]. With one exception the sequences formed a unique genetic group, unrelated to any of the clades or genotypes known at the time. They differed by up to 7 % in the N-gene and 6.9 % in the H gene from any other recent isolates. These strains were assigned to a new clade H. Eleven of 13 strains found in north- and southeast China were closely related differing by no more than 1.5% on a nucleotide level of both H and N genes. The two remaining clade H isolates (Beijing,PRC/94, Hunan.PRC/94) differed from each other and from the other Chinese viruses by at least 2 % in both H and N genes (Fig. 4). Beijing.PRC/94 was later assigned to a different genotype (H2). Between 1995-1999 more clade H isolates were obtained from the provinces of Hunan, Hainan (southeast China), Shandong, Anhui (east China) [61] and Liaoning (northeast China) [62]. The strains analysed by Liffick et al. [61] clustered with

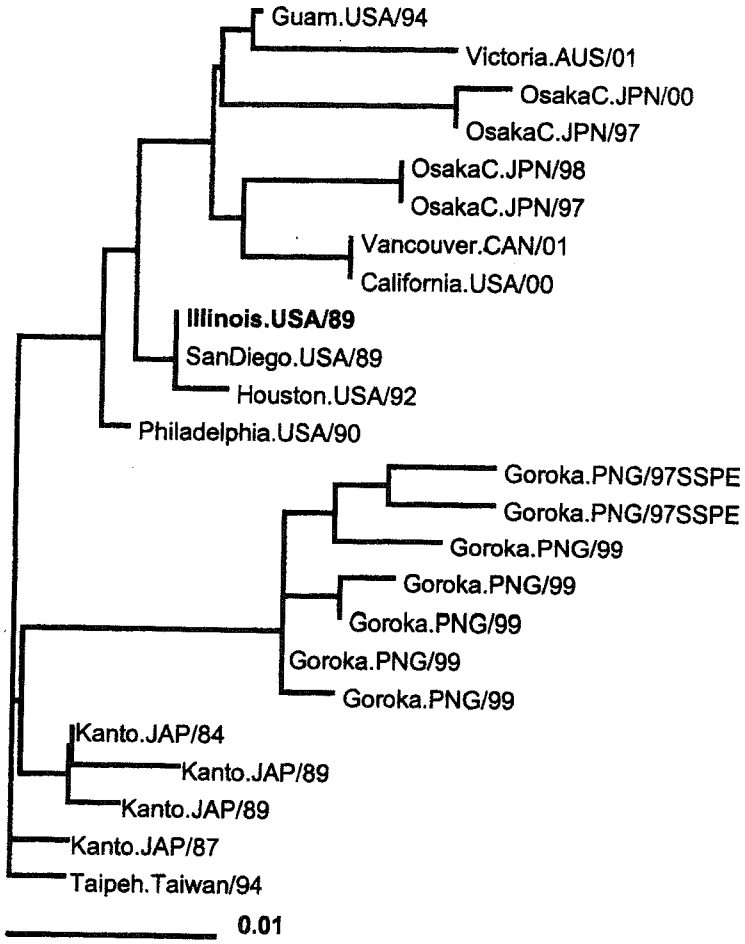

Figure 3: Phylogenetic tree of all D3 strains from Genbank based on 385 C-terminal nucleotides of the N-gene. ClustalX has been used to calculate the tree. To reduce redundancy, isolates from the same place and the same year with identical sequences are presented only once. The province or city, country and year of isolation are given. The reference strain is shown in bold.

all clade H strains of the previous study, except Beijing.PRC/94 (Fig. 4). They showed 5.7 to 10.5 % difference in the N-gene and 4.2 to 6.4 % difference in the H gene compared to strains of other clades, justifying the new clade H.

The only other strains that have been isolated in China (Shandong.PRC/93/5, ChgChina94) belonged to clade A [59,62] and were virtually identical to vaccine strains. Although clade A strains may have been the predominant before the introduction of vaccination in 1965 it is surprising that these viruses would survive ever since with minimal mutations.

These phylogenetic studies showed that a distinct clade of viruses with a considerable sequence diversity was indigenous in east China, indicating that many clade H variants co-circulated and that transmission was still endemic at the time in China.

When the first MV sequences from Vietnam (1998) were reported it became clear that clade H viruses were not only circulating in China [61]. The sequence divergence between the Chinese and the Vietnamese clade H strains was at least 3.5 % and 2.5 % for the

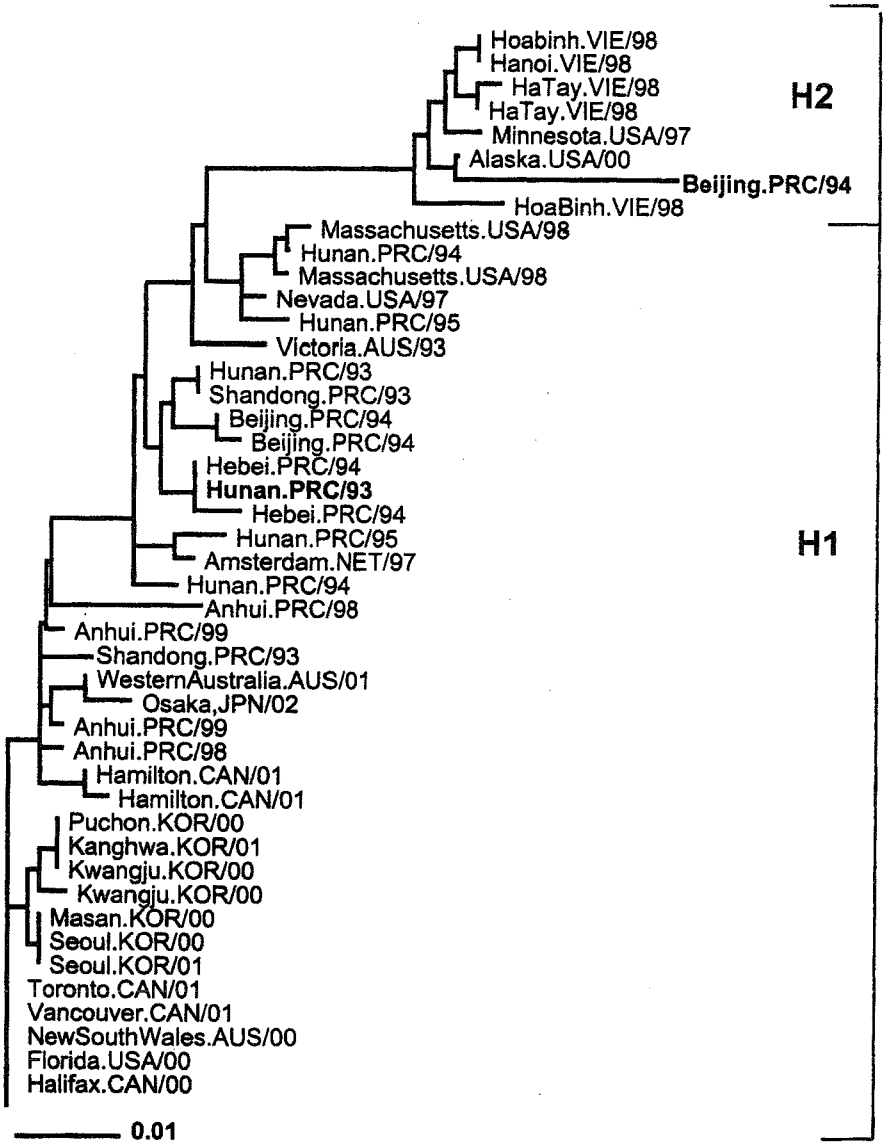

Figure 4: Phylogenetic tree of all clade H viruses from Genbank based on the 450 C-terminal nucleotides of the N-gene, (for further details see also legend to Fig. 3).

HVR of N and the complete H gene, respectively. This difference was estimated sufficient to define two genotypes H1 and H2. The H1 group contains all clade H strains from China except for Beijing.PRC/94 which together with the Vietnamese strains forms the genotype H2 (Fig. 4). Sequence variability within the Vietnamese strains isolated the same year (1998) in the northern part of the country, was 0-1.8 % in N and 0.2-1.1 % in the H gene. This is suggestive of an endemic co-circulation of several H2 variants at the time.

In South-Korea a national immunisation programme was initiated in 1985 [60]. As a result the number of reported measles cases dropped to a record low of <2000 cases. Despite the high coverage a major

outbreak with at least 32000 cases occurred in 2000. Isolates from 4 cities were sequenced [60] and assigned to the Chinese genotype H1 (Fig. 4). Despite their diverse origins, isolates showed only a limited genetic variability of <0,2 % over the complete H and N genes. One year later, very similar viruses continued to be isolated in South-Korea [60]. This suggests that the epidemic may have originated from an index case infected by a virus imported probably from China [7]. It is possible that a similar virus was imported during the following season. Alternatively, the sustained circulation of the same virus could also suggest that the number of susceptibles was sufficient to support endemic transmission of the virus.

Some H1 and H2 have also been isolated from unrelated cases in the US (1997, 2001) [7]. Since measles was no longer indigenous in the US, these viruses must have been imported from other countries, even if their origin was not always independently confirmed. Whenever the source became known, viruses were traced back to patients travelling from either China, Vietnam or Korea. Strains imported from China or Korea were of genotype H1, whereas strains imported from Vietnam were of genotype H2, reflecting the genotype distribution in these countries. Interestingly, an H1 strain isolated in Washington 2001 and originating from China was closely related to Korean isolates from the same year, further suggesting that the virus that caused the 2001 epidemic in Korea was imported from China. H1 strains were also isolated in the UK [62], the Netherlands [36], Australia [72] and Canada [53]. In most cases China was thought to be the source of importation. Epidemiological data suggest that an H1 strain isolated in Germany (Berlin, 2001) was most likely to be imported from Mongolia (personal communication, Dr S. Santibanez, Berlin).

7. INDIAN SUBCONTINENT

Surveillance of measles is limited on the Indian Subcontinent and only a few viruses directly obtained from this vast region have been characterised. Phylogenetic data from US isolates that were epidemiologically traced back to the region have partially filled this gap. The earliest sequencing information comes from two strains collected in Karachi, Pakistan 1989 (Genbank; [36]). These viruses as well as two strains imported from Pakistan to the US in 1995 [69] and 2001 [7] belonged to genotype D4, which was also found in neighbouring Iran [25]. The D4 genotype has also been found during a field investigation of MV strains in Nepal [36]. One of five isolates collected in 4 different locations (Katmandu, Janakpur, Pokhara, Hetauda) in Nepal during 1999 clustered with the isolates from Karachi (Pakistan) and the D4 reference strain MVi/Montréal.CAN/89. The other isolates formed a distinct cluster with an average nucleotide difference of 4.0 % to 7.0 % (of the HVR of N gene) with other established D genotypes. The genetic distance with other clades was even larger. These viruses were therefore assigned to the new genotype D8. The co-circulation of 2 genotypes and the remarkable HVR variability of 3.9% among the D8 isolates collected within a period of 4 weeks indicated that MV circulation in Nepal was still endemic.

Three viruses imported from India to the US [7] and the Netherlands [36] were shown to correspond to D4, before the first sequences of viruses directly collected in India were reported. During 1996-1998, eleven samples from sporadic and outbreak-associated cases were collected in the city of Pune, Maharashtra [73]. This study confirmed the apparent predominance of D4 strains (6 of 11) in India. Furthermore, three isolates were closely related to the D8 viruses previously reported from Nepal. Both the D4 and the D8 strains each formed two distinct phylogenetic clusters. The simultaneous co-circulation of at least four different lineages within several weeks of 1997 in Pune is reminiscent of the multiple chains of endemic transmission in Ibadan, Nigeria [38]. Interestingly two isolates from Pune were assigned to genotype A. Besides four nucleotides difference in the complete H gene of one isolate both viruses were completely identical with both the complete H and the C-terminal part of the N gene of the Edmonston-Zagreb vaccine strain currently used in India [73]. Although, it is difficult to exclude the circulation of wild-type clade A viruses in India, the reisolation of a vaccine strain or a laboratory contamination must be seriously considered [11].

The D4 strains from Pune where closely related both to the isolate from Nepal and the strains imported from India to the US. D4 viruses also seem to have a wide distribution in Eastern and Southern Africa (Kenya, Zimbabwe, Namibia, South-Africa) [74]. Therefore it is an interesting question where the D4 virus has originally evolved. In any case this virus has also been found in Russia and Australia [25,72]. Similarly, the D8 genotype has been found to be prevalent in parts of East-Africa (e.g. Ethopia), although its distribution seems to be more limited [36, 74]. The UK 1994 virus is more closely related to the Indian than to the

Nepalese or the East African D8 viruses and might therefore originate from India. Although early information can be obtained from a global surveillance network, surveillance within each country is necessary to complete the map of genotypes. Considering the size of the Indian Subcontinent, present phylogenetic information is largely insufficient. A cost effective non-sequencing genotyping method would possibly facilitate phylogenetic studies on this continent.

8. MALAYSIA, INDONESIA, EAST TIMOR, PAPUA NEW GUINEA, SINGAPORE

The only two viruses belonging to clade G had been found in the US in 1983 and the lineage was thought to be extinct [39]. However a clade G virus was isolated in 1997 from an immuno-compromised child from Indonesia referred to a Dutch hospital [57]. Since the nucleotide difference between the isolate from Amsterdam and the two early clade G viruses was 3.7% and 3.9 % for H and N gene respectively, the authors assigned the isolate to a new genotype G2 (Fig. 5). Direct evidence of G2 activity came soon afterwards from Indonesia and Malaysia [58]. The complete N and H genes of a 1999-isolate from each country were sequenced and both isolates were members of the proposed new genotype G2.

In 1999, a virus imported from East Timor caused an outbreak in a refugee camp in Victoria, Australia [56]. Although no epidemiological link could be established between this outbreak and another one in Northern Queensland, the nucleotide diversity of the HVR was very limited among all isolates. The viruses were closest to the G2 genotype but differed nonetheless by 2,6 % of the N-gene from the reference strain of this genotype. Partial H sequences showed up to 2,1% nucleotide divergence compared to the G2 reference strain. Therefore, it was proposed to assign these Autralian isolates to another new genotype G3. If until recently clade G was thought to be extinct, this clade has now expanded to three distinct genotypes (Fig. 5).

Another new genotype, D9, has been discovered in Indonesia. The reference strain of this new genotype was isolated in Australia from a measles patient traveling from Bali to Australia [56].

Also the first sequences from Papua New Guinea have been reported recently [68]. After phylogenetic analysis 11 wt isolates where shown to form a distinct cluster within genotype D3. Although isolated in the same year (1999) these strains showed a significant diversity (Table 1). Two SSPE isolates from the same country

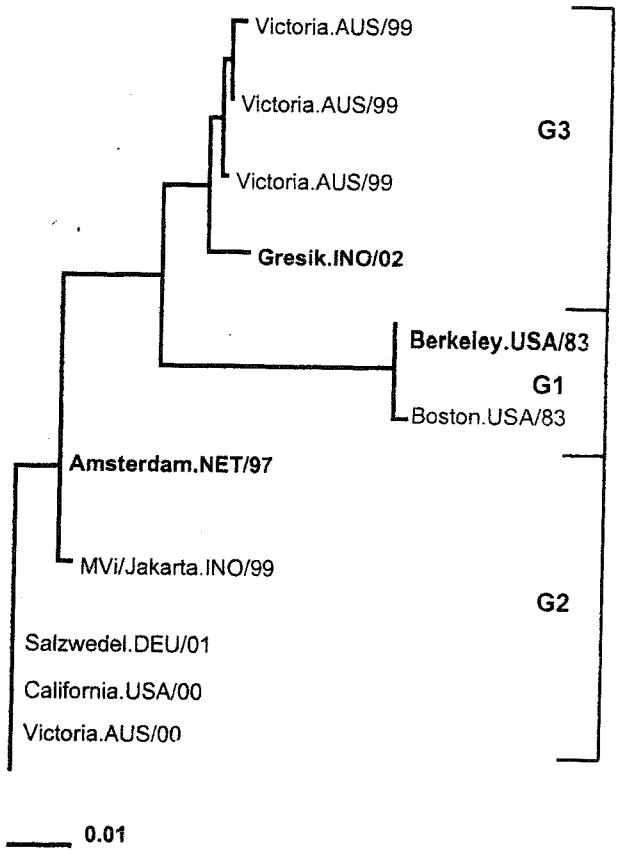

Figure 5: Phylogenetic tree of all clade G strains from Genbank based on the 450 C-terminal nucleotides of the N-gene, (for further details see also legend to Fig. 3).

also clustered with genotype D3. Altogether these data suggest that D3 is the indigenous genotype in Papua New Guinea.

An import from Singapore to Canada was assigned to genotype H1, suggesting the circulation of this genotype in Singapore, which has a strong chinese community [53].

9. RUSSIA AND TURKEY

Only a small number of isolates from the Russian Federation have been sequenced. 6 isolates from local outbreaks in Moscow (1988) and Siberia (1996) were assigned to clade A [45]. Also Jin et al. [75] reported the isolation of a Russian clade A virus from 1988. In

Table 1:

Genotype distribution and nucleotide sequence variability (HVR) in recent outbreaks in Asian and other countries.

Country	Year	Genotype	Variability (%)	Reference
China	1994	**H1**(n=12) / **H2** (n=1) / **A** (n=1)	3.5 / - / -	[59]
India	1996-1998	**A** (n=2) / **D4** (n=5) / **D8** (n=3)	0.0 / 0.4 / 1.1	[73]
Japan	1998	**D3** (n=11) / **D5** (n=2)	0.0 / 0.4	[67]
Japan	2000	**D3** (n=1) / **D5** (n=9)	- / 0.6	[67]
Korea	2000	**H1** (n=8)	0.7	[60]
Nepal	1999	**D4** (n =1) / **D8** (n=4)	- / 3.9	[36]
Pakistan	1989	**D4** (n=2)	1.0	[36]
Papua New Guinea	1999	**D3** (n=11)	1.0	[61]
Vietnam	1998	**H2** (n=17)	1.8	[61]
Burkina Faso	2001	**B3** (n=58)	1.5	[82]
Luxemburg	1996	**D6** (n=9)	0.2	[4]
Nigeria	1998	**B3** (n=31)	4.6	[38]

addition to these clade A isolates a single D6 virus (Siberia, 1997) [45] and D4 viruses were found in Russia [25]. Before concluding that wt clade A viruses dominate in Russia, as is suggested by these data, the reisolation of vaccine strains must be excluded. Ring-vaccination as a response to outbreaks is conducive to reisolation of vaccine virus.

The only MV sequence information from Turkey comes from an isolate of a SSPE patient from 1995. The virus resembles most to a D4 isolate from South Africa identified in 1994 [76]. The nucleotide difference is nevertheless 2,5 % over nt 1281-1595 of the N gene [45]. This suggests that the present isolate may actually represent a new phylogenetic lineage.

10. DISCUSSION

With six new genotypes (D8, D9, H1, H2, G2, G3) discovered during the past five years, phylogenetic analysis of field isolates from Asia has considerably increased the overall diversity of MV strains. Yet from a number of Asian countries sequence information is still missing (Fig. 6). For some countries, as the Philippines or Singapore the only sequence information available comes from strains imported into the US or Canada [7,53,69]. Imported viruses as witnesses of strains and genotypes of the country of origin, are useful in particular as long as local sequence information is limited.

During the different phases of measles control the diversity of circulating strains provides an indication of the number of co-circulating strains, i.e. the number of independent chains of transmission and population immunity. A high sequence diversity suggests endemic transmission, supported by an "unlimited" reservoir of susceptibles. As measles control progresses within an area, chains of transmission are interrupted and indigenous strains loose their diversity. In countries with a population immunity high enough (>95%) to interrupt virus circulation, imported viruses can cause small or large epidemics depending on the numbers of susceptibles that have again accumulated. During an epidemic, viruses have a more limited sequence diversity (Table 1). For instance, during the 1996

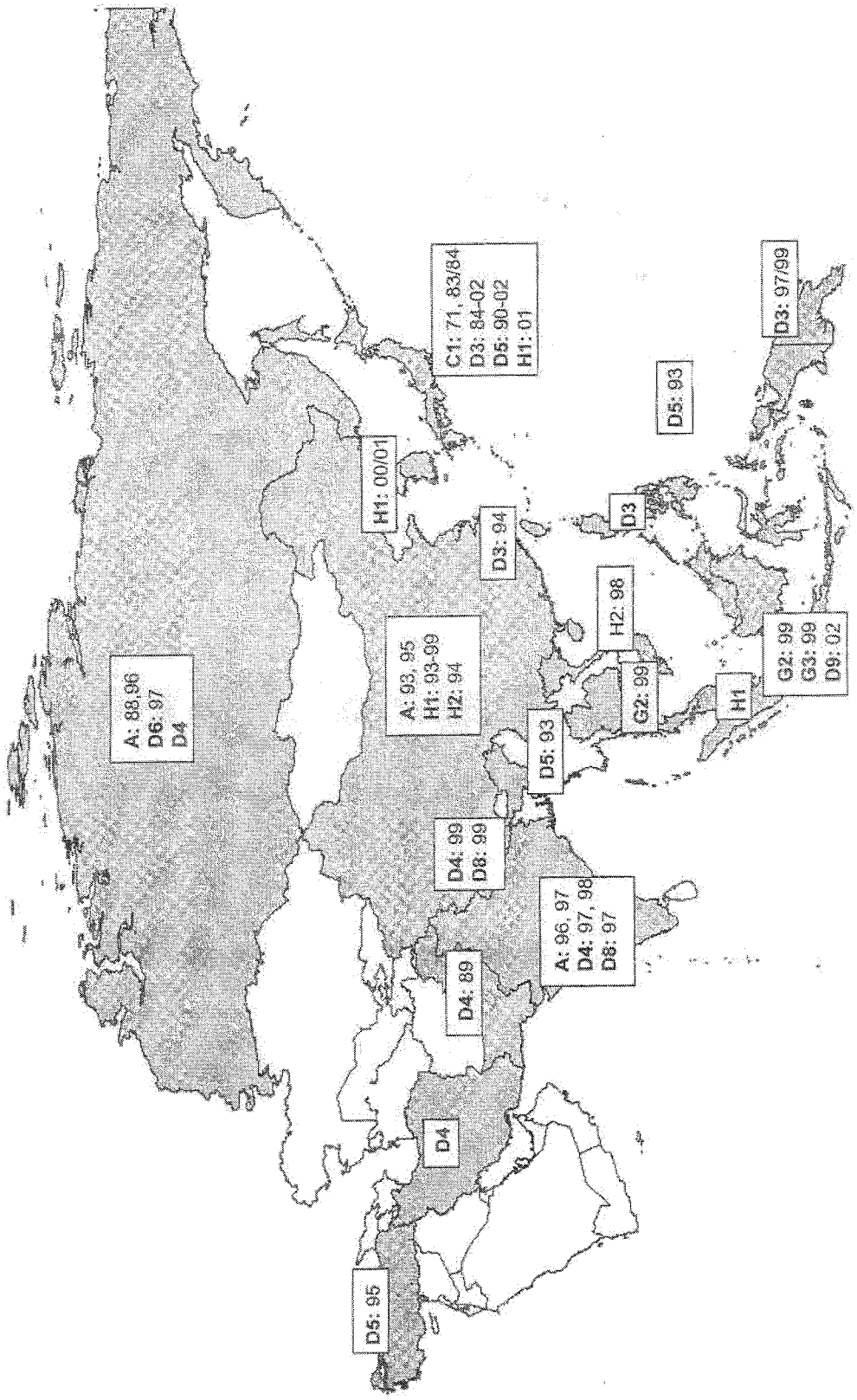

Figure 6: MV genotype map of the Asian continent. Shaded countries with genotype information and date of isolation if available.

outbreak in Luxembourg schools the diversity was 0.2% (HVR of N; n=9; [4]). In contrast, strains collected in 1998 within three weeks in the city of Ibadan, Nigeria, were much more diverse (4.6%; n=31; [38]). In the Houët province, Burkina Faso >400 cases were reported in 2001 despite recent national immunization days. Compared to the observations in Nigeria, the sequence variability was much lower (1,5 %, n=58), suggesting progress from endemic to epidemic transmission in Burkina [75].

The review of sequence diversity in Asia, shows that many countries continue to face endemic MV circulation. The high sequence diversity of clade H strains in China and Vietnam [59,61,62], of D3 strains in Papua New Guinea (1999) [68] and the co-circulation of different genotypes in India and Nepal [36,73] suggest many independent chains of endemic transmission (Table 1).

Japan experienced several rounds of dis- and replacement of genotypes (C1, D3, D5) throughout the eighties and nineties [63,65,77]. Total displacement of C1 strains indicates a high level of measles control, following introduction and intensification of vaccination programmes. However the disease re-emerged probably after re-importation because of insufficient population immunity and accumulation of susceptibles. Since genotypes D3 and D5 have been detected more or less continuously since the mid 80's in Japan low level of endemic transmission may still persist. However, the Japanese D3 strains of the late nineties are more closely related to those from the 1989-1991 epidemic in the US than to those from the early nineties (Fig. 3). Therefore the more recent D3 isolates may also be a result of (multiple) importation.

Genotype displacement has been observed as a result of successful vaccination in a number of countries including the US [3], Argentina [5] and others. Surprisingly, the above phenomenon has also been seen in countries with less optimal control measures such as Spain [51] and Germany [43], with the possibility that some viruses may be fitter than others [23]. In this context, the antigenic significance of the additional glycosilation site in the H protein (which is the main target of neutralising antibodies) of D3 and D5 strains in comparison to C1 strains [64] would be of interest.

Measles virus genotypes have evolved in relative geographic seclusion To understand the geographic distribution of genotypes before vaccination (i.e. indigenous *sensu strictu)* is one of the challenges of measles molecular epidemiology. Although indigenous genotypes of some countries seem to be clear, measles control measures blur the pre-vaccination strain distribution (Fig. 6). Endemic regions are usually dominated by one or two genotypes. The indigenous strain is H1 in China and H2 in Vietnam [61]; these viruses seem to have no other hub in the world. In Korea, recent isolates are very homogenous and most probably imported from China [60]. The diversity of co-circulating D3 strains in 1999 is compatible with this genotype being indigenous in Papua New Guinea [68], although variants of this genotype have been found in other East Asian locations as well as imports to the US, Canada and Australia (Fig 6) [7,53,72]. In Japan, C1 was predominant before the pre-vaccine era, but single isolates were also found in Europe during the seventies. For most other Asian countries, one can only speculate about the indigenous genotypes. In India, transmission has always been endemic; therefore the prevaccination indigenous genotype should still be prevalent. However, the dearth of sequence information limited to a single city, and the co-circulation of different genotypes (A, D4, D8) in this city complicates the identification of indigenous genotypes [73]. The same accounts for Nepal [36].

Clade A viruses have been found in the US (Edmonston Wild Type, 1954) [37], the Czech Republic (1960) [45], Denmark [78] and Finland [39] at least until the late sixties, suggesting a wide distribution of this clade before the introduction of routine vaccination in the 1970's [79]. If this was the case, clade A viruses have been evicted from virtually all countries after the introduction of routine vaccination.

Yet, clade A strains have been found sporadically during the eighties and nineties, in some countries including the UK (1993 [62, 80]) and South Africa (1988/1989/1995 [76]). The recent isolations of clade A strains throughout Asia (China, India and Russia [45, 59, 62, 73]) confirm that the geographic distribution and diversity of clade A isolates do not follow the pattern of any other genotype. Fig. 7 shows the virtual identity of clade A isolates related to both the vaccine strains and Edmonston WT. Based on 350 nucleotides at the C-terminal end of N gene, two Russian isolates are identical to the Leningrad-16 vaccine strain and Edmonston WT strain. Two other Russian clade A strains are identical to the Shanghai-191, Schwarz, Moraten and Edmonston Bb vaccine strain. The Chinese clade A isolate has one point mutation compared to the Edmonston wt strain as well as 2 point mutations compared to the Shanghai-191 and the Changchun-47 vaccine strain. The two Indian wild-type isolates are identical to the Edmonston wt and the

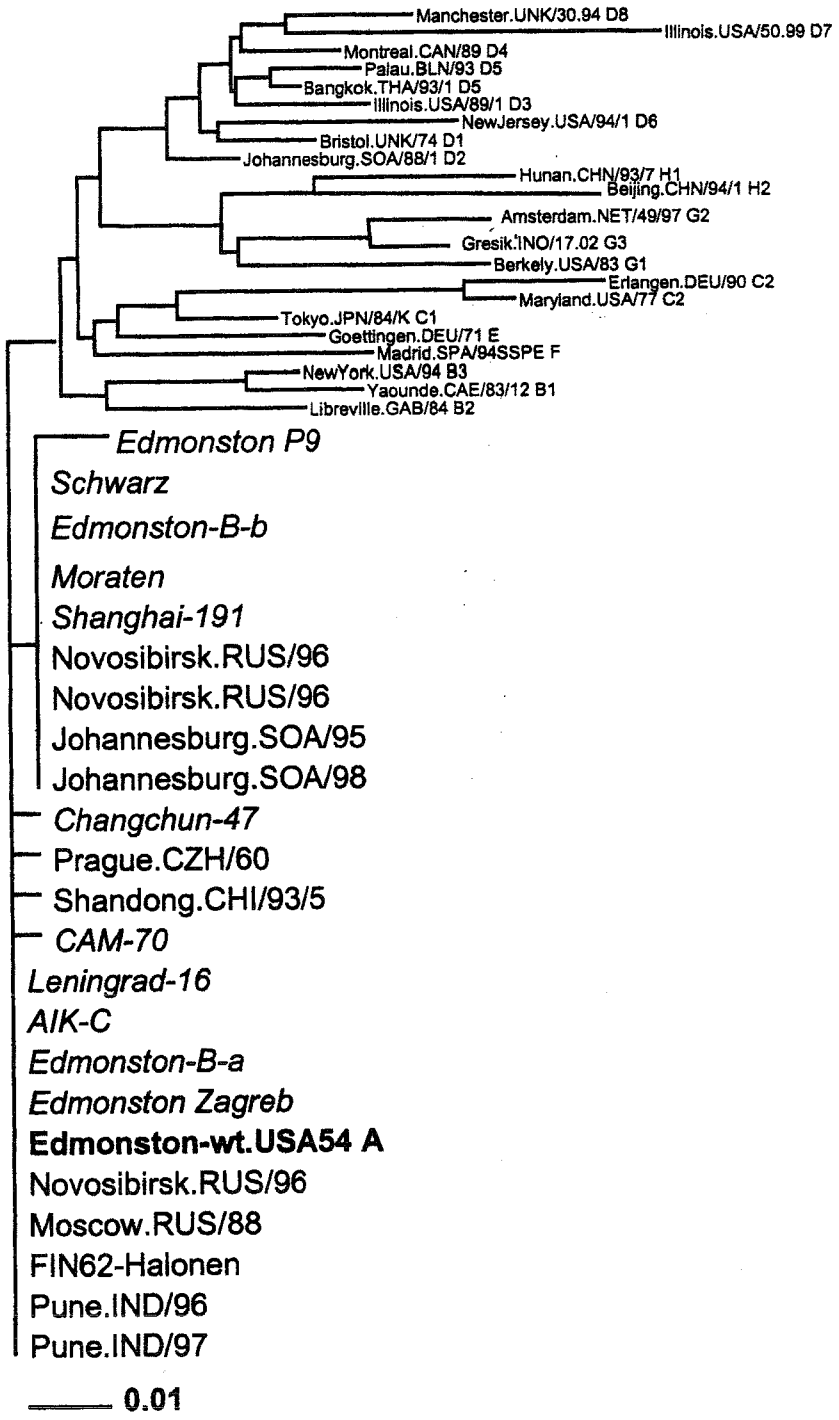

Figure 7: Phylogenetic tree of all vaccine strains and some clade A field isolates based on the 340 C-terminal nucleotides of the N-gene. Reference strains of the different genotypes are also included. Vaccine strains are shown in Italic.

Edmonston Zagreb. Fig. 7 shows furthermore that clade A viruses reveal a minimal sequence diversity irrespective of the time and place of isolation. These observations could be most easily reconciled by reisolation or laboratory contamination by vaccine strain.

Sequence diversity between strains isolated in different geographical locations and/or at different time points can be used to follow chains of transmission and circulation pathways, and provide important guidelines for control efforts.

So far strain differences have not be related to enhanced pathogenicity and complication rates. These are usually explained by host or socio-economic factors, but with the increasing genetic diversity of MV viral factors should nevertheless be considered. Regarding the clear predominance of certain genotypes in regions with a high mortality rate, like Sub-Saharan Africa (B3) or China (H1), it would be interesting to investigate the virulence of these strains. Genetic characterisation of MV strains may not only be useful to follow epidemiological routes of transmission but could also contribute to a better understanding of the pathophysiology of the virus.

Note added in proof: Recent additional sequencing data show that the SSPE isolate from Turkey (1995) belongs to genotype D5 (personal communication, Dr. S. Santibanez, Berlin).

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12. REFERENCES

[1] Mossong, J., and Muller, C.P., 2000, Epidemiol Infect, 124, 273-8.

[2] Anderson, R.M., and May, R.M., 1985, J Hyg (Lond), 94, 365-436.

[3] Rota, J.S., Heath, J.L., Rota, P.A., King, G.E., Celma, M.L., Carabana, J., Fernandez-Munoz, R., Brown, D., Jin, L., and Bellini, W.J., 1996, J Infect Dis, 173, 32-7.

[4] Hanses, F., van Binnendijk, R., Ammerlaan, W., Truong, A.T., de Rond, L., Schneider, F., and Muller, C.P., 2000, Arch Virol, 145, 541-51.

[5] Barrero, P.R., Zandomeni, R.O., and Mistchenko, A.S., 2001, Arch Virol, 146, 815-23.

[6] van den Hof, S., Meffre, C.M., Conyn-van Spaendonck, M.A., Woonink, F., de Melker, H.E., and van Binnendijk, R.S., 2001, Emerg Infect Dis, 7, 593-7.

[7] Rota, P.A., Liffick, S.L., Rota, J.S., Katz, R.S., Redd, S., Papania, M., and Bellini, W.J., 2002, Emerg Infect Dis, 8, 902-8.

[8] WHO - World Health Organization, 1999, Geneva, 98-104.

[9] World Health Assembly, 1989, Resolution No. (Wha)42.32, in Executive summary.

[10] CDC - Centers for Disease Control and Prevention, 1997, MMWR Morb Mortal Wkly Rep, 46, 1-20.

[11] Muller, C.P., and Mulders, N.M., 2002, The Molecular Epidemiology of Human Viruses, e.K. Thomas Leitner, Kluwer academic Publishers, Boston, 237-272.

[12] Osterhaus, A.D., Groen, J., De Vries, P., UytdeHaag, F.G., Klingeborn, B., and Zarnke, R., 1988, Nature, 335, 403-4.

[13] Visser, I.K., Kumarev, V.P., Orvell, C., de Vries, P., Broeders, H.W., van de Bildt, M.W.,

Groen, J., Teppema, J.S., Burger, M.C., UytdeHaag, F.G., et al., 1990, Arch Virol, 111, 149-64.

[14] Kennedy, S., Smyth, J.A., Cush, P.F., McCullough, S.J., Allan, G.M., and McQuaid, S., 1988, Nature, 336, 21.

[15] Domingo, M., Ferrer, L., Pumarola, M., Marco, A., Plana, J., Kennedy, S., McAliskey, M., and Rima, B.K., 1990, Nature, 348, 21.

[16] Van den Hoogen, B.G., de Jong, J.C., Groen, J., Kuiken, T., de Groot, R., Fouchier, R.A., and Osterhaus, A.D., 2001, Nat Med, 7, 719-24.

[17] Wang, L.F., Yu, M., Hansson, E., Pritchard, L.I., Shiell, B., Michalski, W.P., and Eaton, B.T., 2000, J Virol, 74, 9972-9.

[18] Chua, K.B., Bellini, W.J., Rota, P.A., Harcourt, B.H., Tamin, A., Lam, S.K., Ksiazek, T.G., Rollin, P.E., Zaki, S.R., Shieh, W., Goldsmith, C.S., Gubler, D.J., Roehrig, J.T., Eaton, B., Gould, A.R., Olson, J., Field, H., Daniels, P., Ling, A.E., Peters, C.J., Anderson, L.J., and Mahy, B.W., 2000, Science, 288, 1432-5.

[19] Schrag, S.J., Rota, P.A., and Bellini, W.J., 1999, J Virol, 73, 51-4.

[20] Rima, B.K., Earle, J.A., Baczko, K., ter Meulen, V., Liebert, U.G., Carstens, C., Carabana, J., Caballero, M., Celma, M.L., and Fernandez-Munoz, R., 1997, J Gen Virol, 78, 97-106.

[21] Fayolle, J., Verrier, B., Buckland, R., and Wild, T.F., 1999, J Virol, 73, 787-90.

[22] Truong, A.T., Kreis, S., Ammerlaan, W., Hartter, H.K., Adu, F., Omilabu, S.A., Oyefolu, A.O., Berbers, G.A., and Muller, C.P., 1999, Virus Res, 62, 89-95.

[23] Klingele, M., Hartter, H.K., Adu, F., Ammerlaan, W., Ikusika, W., and Muller, C.P., 2000, J Med Virol, 62, 91-8.

[24] WHO - World Health Organization, 2001, Wkly Epidemiol Rec, 76, 242-7.

[25] WHO - World Health Organization, 2001, Wkly Epidemiol Rec, 76, 249-51.

[26] Mori, T., Sasaki, K., Hashimoto, H., and Makino, S., 1993, Virus Genes, 7, 67-81.

[27] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G., 1997, Nucleic Acids Res, 25, 4876-82.

[28] Kumar, S., Tamura, K., and Nei, M., 1994, Comput Appl Biosci, 10, 189-91.

[29] Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M., 2001, Mega2: Molecular Evolutionary Genetics Analysis Software.

[30] Felsenstein, J., 1993, Phylogeny Inference Package (Phylip).

[31] Saitou, N., and Nei, M., 1987, Mol Biol Evol, 4, 406-25.

[32] Nei, M., and Kumar, S., 2000, Molecular Evolution and Phylogenetics, Oxford University Press.

[33] Felsenstein, J., 1981, J Mol Evol, 17, 368-76.

[34] Lake, J.A., 1987, Mol Biol Evol, 4, 167-91.

[35] EPI - Expanded Program on Immunization, 1998, Wkly Epidemiol Rec, 73, 265-9.

[36] Truong, A.T., Mulders, M.N., Gautam, D.C., Ammerlaan, W., de Swart, R.L., King, C.C., Osterhaus, A.D., and Muller, C.P., 2001, Virus Res, 76, 71-8.

[37] Enders, J.F., and Peebles, T.C., 1954, Proc Soc Exp Biol Med, 86, 277-286.

[38] Hanses, F., Truong, A.T., Ammerlaan, W., Ikusika, O., Adu, F., Oyefolu, A.O., Omilabu, S.A., and Muller, C.P., 1999, J Gen Virol, 80, 871-7.

[39] Rota, P.A., Bloom, A.E., Vanchiere, J.A., and Bellini, W.J., 1994, Virology, 198, 724-30.

[40] WHO-World Health Organization, 2003, Wkly Epidemiol Rec, 78, 229-232.

[41] Outlaw, M.C., Jaye, A., Whittle, H.C., and Pringle, C.R., 1997, Virus Res, 48, 125-31.

[42] El Mubarak, H.S., Van De Bildt, M.W., Mustafa, O.A., Vos, H.W., Mukhtar, M.M., Groen, J., el Hassan, A.M., Niesters, H.G., Ibrahim, S.A., Zijlstra, E.E., Wild, T.F., Osterhaus, A.D., and De Swart, R.L., 2000, J Clin Microbiol, 38, 987-91.

[43] Santibanez, S., Tischer, A., Heider, A., Siedler, A., and Hengel, H., 2002, J Gen Virol, 83, 2699-708.

[44] Dahl, L., Christensen, L.S., Schoeller, S., Westh, H., and Plesner, A.M., 2000, Apmis, 108, 267-72.

[45] Santibanez, S., Heider, A., Gerike, E., Agafonov, A., and Schreier, E., 1999, J Med Virol, 58, 313-20.

[46] Barrero, P.R., de Wolff, C.D., Passeggi, C.A., and Mistchenko, A.S., 2000, J Med Virol, 60, 91-6.

[47] Baumeister, E., Siqueira, M.M., Savy, V., and Friedrich, F., 2000, Acta Virol, 44, 169-74.

[48] Bilkis, M.D., Barrero, P.R., and Mistchenko, A.S., 2000, Epidemiol Infect, 124, 289-93.

[49] Canepa, E., Siqueira, M.M., Hortal, M., and Friedrich, F., 2000, Acta Virol, 44, 35-9.

[50] Siqueira, M.M., Castro-Silva, R., Cruz, C., Oliveira, I.C., Cunha, G.M., Mello, M., Rota, P.A., Bellini, W.J., and Friedrich, F., 2001, J Med Virol, 63, 299-304.

[51] Rima, B.K., Earle, J.A., Yeo, R.P., Herlihy, L., Baczko, K., ter Meulen, V., Carabana, J., Caballero, M., Celma, M.L., and Fernandez-Munoz, R., 1995, J Gen Virol, 76, 1173-80.

[52] Taylor, M.J., Godfrey, E., Baczko, K., ter Meulen, V., Wild, T.F., and Rima, B.K., 1991, J Gen Virol, 72, 83-8.

[53] Tipples, G.A., Gray, M., Garbutt, M., and Rota, P.A., 2002, J Infect Dis, Submitted

[54] Cattaneo, R., Schmid, A., Spielhofer, P., Kaelin, K., Baczko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B.K., Udem, S.A., and et al., 1989, Virology, 173, 415-25.

[55] Chibo, D., Riddell, M.A., Catton, M.G., and Birch, C.J., 2002, Emerg Infect Dis, 8, 735-737.

[56] Chibo, D., Riddell, M., Catton, M., Lyon, M., Lum, G., and Birch, C., 2003, Virus Res, 91, 213-21.

[57] De Swart, R.L., Wertheim-van Dillen, P.M., van Binnendijk, R.S., Muller, C.P., Frenkel, J., and Osterhaus, A.D., 2000, Lancet, 355, 201-2.

[58] Rota, P.A., Liffick, S., Rosenthal, S., Heriyanto, B., and Chua, K.B., 2000, Lancet, 355, 1557-8.

[59] Xu, W., Tamin, A., Rota, J.S., Zhang, L., Bellini, W.J., and Rota, P.A., 1998, Virus Res, 54, 147-56.

[60] Na, B.K., Lee, J.S., Shin, G.C., Shin, J.M., Lee, J.Y., Chung, J.K., Ha, D.R., Lee, J.K., Ma, S.H., Cho, H.W., Kang, C., and Kim, W.J., 2001, Virus Res, 81, 143-9.

[61] Liffick, S.L., Thi Thoung, N., Xu, W., Li, Y., Phoung Lien, H., Bellini, W.J., and Rota, P.A., 2001, Virus Res, 77, 81-7.

[62] Jin, L., Sun, Y.J., Ge, L., and Brown, D.W., 1998, Epidemiol Infect, 121, 691-7.

[63] Yamaguchi, S., 1997, J Med Virol, 52, 113-20.

[64] Sakata, H., Kobune, F., Sato, T.A., Tanabayashi, K., Yamada, A., and Sugiura, A., 1993, Microbiol Immunol, 37, 233-7.

[65] Nakayama, T., Mori, T., Yamaguchi, S., Sonoda, S., Asamura, S., Yamashita, R., Takeuchi, Y., and Urano, T., 1995, Virus Res, 35, 1-16.

[66] Takahashi, M., Nakayama, T., Kashiwagi, Y., Takami, T., Sonoda, S., Yamanaka, T., Ochiai, H., Ihara, T., and Tajima, T., 2000, J Med Virol, 62, 278-85.

[67] Kubo, H., Iritani, N., and Seto, Y., 2003, J Med Virol, 69, 273-278.

[68] Miki, K., Komase, K., Mgone, C.S., Kawanishi, R., Iijima, M., Mgone, J.M., Asuo, P.G., Alpers, M.P., Takasu, T., and Mizutani, T., 2002, J Med Virol, 68, 105-12.

[69] Rota, J.S., Rota, P.A., Redd, S.B., Redd, S.C., Pattamadilok, S., and Bellini, W.J., 1998, J Infect Dis, 177, 204-8.

[70] Rota, P.A., Liffick, S.L., Rota, J.S., Katz, R.S., Redd, S., Papania, M., and Bellini, W.J., 2002, Emerg Infect Dis, 8, 902-908.

[71] Kubo, H., Iritani, N., Murakami, T., and Haruki, K., 2002, Jpn J Infect Dis, 55, 177-9.

[72] Chibo, D., Birch, C.J., Rota, P.A., and Catton, M.G., 2000, J Gen Virol, 81 Pt 10, 2511-8.

[73] Wairagkar, N., Rota, P.A., Liffick, S., Shaikh, N., Padbidri, V.S., and Bellini, W.J., 2002, J Med Virol, 68, 611-4.

[74] Nigatu, W., Jin, L., Cohen, B.J., Nokes, D.J., Etana, M., Cutts, F.T., and Brown, D.W., 2001, J Med Virol, 65, 373-80.

[75] Jin, L., Richards, A., and Brown, D.W., 1996, Mol Cell Probes, 10, 191-200.

[76] Kreis, S., Vardas, E., and Whistler, T., 1997, J Gen Virol, 78, 1581-7.

[77] Katayama, Y., Shibahara, K., Kohama, T., Homma, M., and Hotta, H., 1997, J Clin Microbiol, 35, 2651-3.

[78] Christensen, L.S., Scholler, S., Schierup, M.H., Vestergaard, B.F., and Mordhorst, C.H., 2002, Apmis, 110, 113-22.

[79] Rota, J.S., Wang, Z.D., Rota, P.A., and Bellini, W.J., 1994, Virus Res, 31, 317-30.

[80] Outlaw, M.C., and Pringle, C.R., 1995, Virus Res, 39, 3-11.

[81] Takeuchi, K., Miyajima, N., Kobune, F., and Tashiro, M., 2000, Virus Genes, 20, 253-7.

[82] Mulders, M.N., Nebie, Y.K., Fack, F., Kapitanyuk, T., Sanou, O., Valéa, D.C., Muyembe-Tamfum, J., Ammerlaan, W., and Muller, C.P., 2003, J Infect. Dis, 187 (suppl.1), S 277-82.

X. Génotypage du virus de la rougeole par PCR multiplex

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Résumé de l'article

L'épidémiologie moléculaire est un outil très important pour la surveillance de la rougeole. Un protocole standardisé, fondé sur le séquençage partiel du génome viral a été élaboré par l'OMS pour attribuer chaque souche à un génotype précis. Cependant cette méthode s'avère peu adaptée à un grand nombre d'échantillons et requiert des facilités de séquençage.

C'est la raison pour laquelle nous avons développé une méthode de génotypage plus simple et plus rapide pour faire la distinction entre les clades et génotypes du virus de la rougeole considérés actifs (A, B3.1.-B3.2., C2, D2-D9, G2-G3, et H1-H2). Cette méthode est basée sur 6 PCR multiplex différentes: une qui identifie le clade (A,B,C,D,G,H) et cinq autres pour faire la distinction entre les différents génotypes à l'intérieur d'un clade. Chaque réaction contient un mélange d'amorces sens spécifiques des différents clades ou génotypes, ainsi qu'une amorce antisens universelle pour tous les génotypes. La spécificité des amorces est due à la présence de nucléotides spécifiques d'un clade ou génotype comme base terminale 3'. Ces nucléotides ont été identifiés par alignement de toutes les séquences C-terminales (450 nucléotides) du gène N publiées avant 2003. Les conditions PCR ont été optimisées de telle sorte que seule l'amorce sens spécifique du génotype testé soit élongée par la polymérase. Puisque les différentes amorces inclues dans les différents mélanges se lient à différents endroits de l'ADN cible, les tailles des fragments d'ADN produits sont caractéristiques d'un clade ou génotype. Pour identifier leur taille, les produits PCR sont séparés par électrophorèse en gel d'Agarose, et comparés à un marqueur d'ADN.

En pratique chaque souche devra d'abord être soumise à la PCR multiplex qui identifie le clade. Dans le cas des souches appartenant aux clades A ou C, la procédure se termine à ce stade puisque ces deux clades ne contiennent qu'un seul génotype actif. Les souches appartenant aux clades B, D, G, et H sont caractérisées davantage dans une deuxième réaction qui fait intervenir les amorces spécifiques des génotypes appartenant au clade

identifié. Vu le grand nombre de génotypes appartenant au clade D (n=8), deux réactions différentes avec des amorces spécifiques de quatre génotypes chacune, sont lancées en parallèle. Cette technique a été établie et validée en utilisant des fragments PCR couvrant toute la partie C-terminale du gène N (450 nucléotides). Au moins trois souches différentes ont été testées pour la plupart des génotypes. En se basant sur toutes les séquences publiées avant 2003, cette technique a une spécificité et une sensitivité théorique de 98.6% et 99.2% respectivement.

Cette méthode permet donc d'identifier les différents génotypes du virus de la rougeole sans séquençage, facilitant ainsi le génotypage d'un grand nombre de souches sauvages, dans un plus grand nombre de laboratoires. Cependant, l'identification de nouveaux génotypes ainsi que la caractérisation de souches qui ne portent pas les nucléotides spécifiques de leur clade ou génotype ne pourront être obtenues que par séquençage.

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Measles Virus Genotyping by Nucleotide-Specific Multiplex PCR

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A simple genotyping method based on multiplex PCR has been developed to discriminate between all active measles virus (MV) clades and genotypes (A, B3.1, B3.2, C2, D2-D9, G2-G3, and H1-H2). The sequencing reaction was replaced by six multiplex PCRs: one to identify the clade and five to identify the respective genotype. Primers were sensitive to clade- and genotype-specific nucleotides and generated fragments of type-specific sizes that were analyzed by conventional agarose gel electrophoresis. On the basis of all published MV sequences, positive and negative predictive values of 99.2% and 98.6% were calculated. Variability in the primer binding sites, which could potentially reduce sensitivity, was very limited among published sequences. As new genotypes are described, additional specific primers can be included in the multiplex PCR with relative ease. Although sequencing remains the "gold standard," the present method should facilitate MV genotyping especially in developing countries and will therefore contribute to enhanced MV control and elimination strategies as recommended by the World Health Organization.

Global measles incidence has dramatically decreased after the introduction of routine vaccination, but endemic circulation persists in many developing countries in Asia and Africa (10, 12). To reduce measles mortality, the World Health Organization (WHO) has renewed its effort for global measles control on the basis of enhanced vaccination and surveillance. Molecular epidemiology of measles virus (MV) has proven very useful for monitoring the efficiency of vaccination campaigns, for monitoring routes of transmission, and for proving local interruption of virus circulation (13).

MV is a monotypic morbillivirus belonging to the family of *Paramyxoviridae*. The negative-stranded nonsegmented RNA genome encodes eight proteins, including the nucleocapsid (N) and the hemagglutinin (H) proteins. Sequence diversity within the complete H gene and the 450 C-terminal nucleotides of the N gene (nucleotides [nt] 1233 to 1682) (11) classifies MV strains into eight clades (A to H) containing 22 different genotypes (A, B1 to B3, C1 and C2, D1 to D9, E, F, G1 to G3, and H1 and H2). Most MV genotypes have a more or less characteristic geographic distribution. Genotypes B1, B2, D1, E, F, and G1 are considered to be inactive (25).

MV sequence information from many (developing) countries with a persistently high measles incidence is limited. A simple genotyping method, without the need for sequencing, could potentially contribute to a more complete picture of MV genotype distribution throughout the world and thus enhance MV control programs. The WHO recommends that the genotype of representative strains should be determined from every outbreak (25).

Few alternative methods, limited to one or a few genotypes, have been described for MV. Samuel et al. developed a method based on a modification of the amplification refractory mutation system to genotype D6 strains (20). Takahashi et al. used restriction fragment length polymorphism to distinguish between genotypes C1, D3, and D5 (22). Here we propose a genotyping strategy based on clade- and genotype-specific multiplex PCR to discriminate between all currently circulating MV genotypes.

MATERIALS AND METHODS

RNA isolation and RT-PCR. Viruses were obtained from the American Type Culture Collection, Manassas, Va. (U01987, clade A [18]); A. D. M. E. Osterhaus, Rotterdam, The Netherlands (AF193513, genotype D4 [24]; AF171232, G2 [24]; AF193512, H1 [24]); A. Tischer, Berlin, Germany (AF474930, D7 [21]); T. Whistler, Johannesburg, South Africa (U64582, D2 [9]); D. Chibo, Victoria, Australia (AF481485, D9 [5]); and P. A. Rota, Atlanta, Ga. (M89921, C2 [1]; U01977, D3 [18]; L46758, D5 [17]; L46750, D6 [17]; AY184217, G3 [4]; AF045217, H2 [26]). The remaining strains were isolated in our laboratory by standard cocultivation on B95a cells (AJ232203, B3.1; AJ232209, B3.2 [7]; AJ250070, D8 [24]) as described previously (7). RNA was extracted from 200 μl of virus culture supernatant (RNeasy Kit; Qiagen, Leiden, The Netherlands) according to the manufacturer's protocol. Specific cDNA of MV nucleoprotein mRNA was synthesized by reverse transcription (RT) with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Merelbeke, Belgium) and primer MVN8 (TTA TAA CAA TGA TGG AGG, nt 1740 to 1722). The cDNA was further amplified by using the primers MVN8 and NP3seq (TTG CTG GTG AGT TAT CCA CAC TTG, nt 973 to 996) to obtain a 768-bp fragment. This was followed by a nested PCR with MVN1 (GAT GGT AAG GAG GTC AGC TGG, nt 1208 to 1228) and MVN7 (TCG GCC TCT CGC ACC TA, nt 1699 to 1683), respectively, to increase sensitivity of detection and specificity.

Clade- and genotype-specific PCR. The product of the MVN1/MVN7-nested PCR of the virus of interest was included as a template (2 μl, 1:100 diluted) into 15 μl of a PCR mixture containing 1.7 mM $MgCl_2$, 1× PCR buffer (Invitrogen), 0.5 mM deoxynucleoside triphosphate, 0.6 U of Platinum *Taq* DNA polymerase (Invitrogen) to perform the cladetyping and genotyping multiplex PCRs (Fig. 1). The same common antisense primer revCG (0.4 μM) (GGGTGTCCGTGTCT GAGCCTTG, nt 1650 to 1629) was used in all reactions. Different combinations

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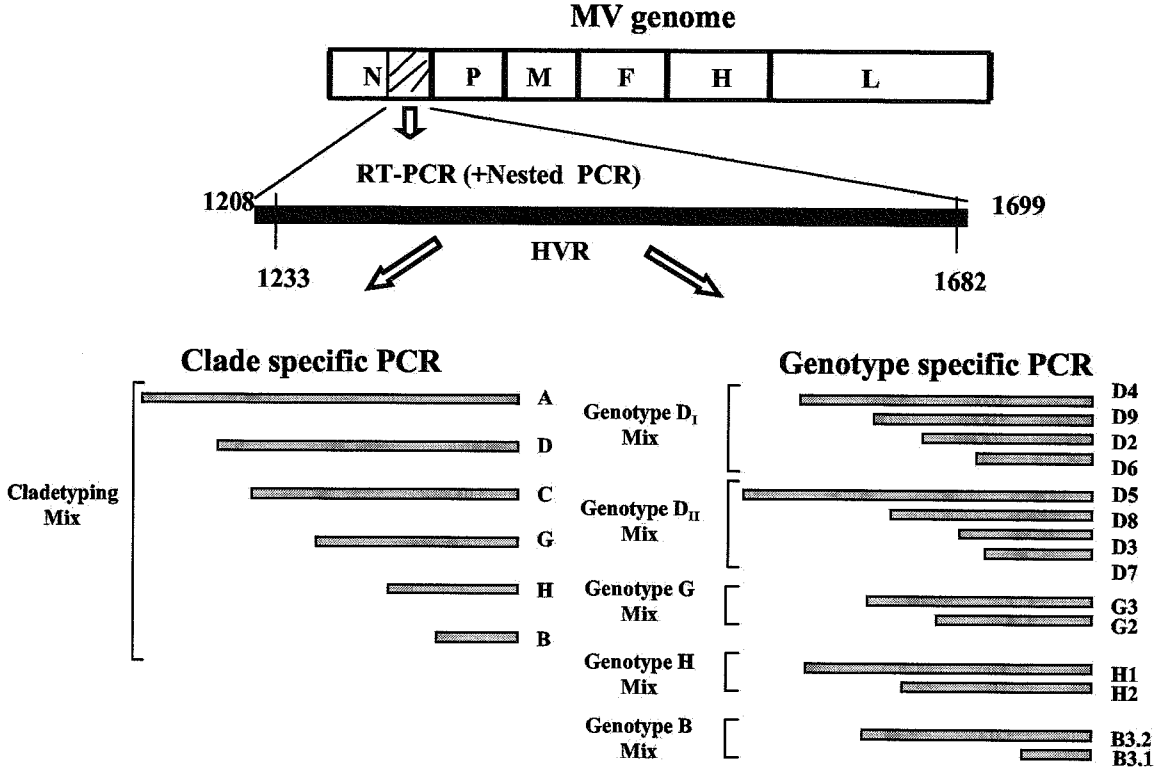

FIG. 1. Strategy for clade- and genotyping of MV by multiplex PCR with type-specific primers. Bars represent relative fragment lengths. N, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; H, hemagglutinin protein; L, large protein.

of sense primers were included into either the cladetyping or the different genotyping multiplex PCR mixtures at the concentrations given in Table 1. After an initial denaturation step at 95°C for 2 min, amplification was performed by using 25 cycles of sequential denaturation (30 s at 95°C), annealing (10 s at 66°C), and elongation (10 s at 72°C). The reaction was carried out on a Mastercycler Gradient (Eppendorf, Hamburg, Germany). PCR fragments were separated in a 3.5% agarose gel (Invitrogen). The fragment size was determined in comparison to the 1-Kb Plus DNA Ladder (Invitrogen).

RESULTS

Clade- and genotype-specific nucleotides. Clade- and genotyping was based on type-specific single nucleotide differences between clades or genotypes of MV. All published sequences of the hypervariable region (HVR) of the MV N gene (nt 1233 to 1682) were aligned and assigned to their respective genotypes by phylogenetic analysis as described previously (23). Sequences belonging to the officially extinct lineages (B1, B2, D1, E, F, and G1), as well as those belonging to genotype C1, which was last detected 10 years ago (2), were removed from the data set. Clade- and genotype-specific nucleotides were identified among the remaining 613 sequences. The number of sequences available for the different genetic groups ranged between 4 (D9 and G3) and 268 (clade D). Figure 2 shows that for most clades at least one nucleotide exists, which was not found in strains of other clades. The nucleotide A1321 was found in all but 1 (2.8%) clade A virus and in 4 (0.7%) of 594 viruses of other clades. The clade G-specific nucleotide (A1486) was also present in one D4 sequence. With the exception of D2, each genotype carried a characteristic nucleotide that was absent in any other genotype of the same clade. The D2-specific nucleotide (A1482) was also found in four viruses belonging to D4 or D6.

Primer design. Both cladetyping and genotyping reactions were based on nucleotide-specific PCR with type-specific sense primers and a common antisense primer (nt 1649 to 1629). Specificity resulted from the type-specific nucleotides of Fig. 2 incorporated as a 3′-terminal base into the respective primers. Under appropriate experimental conditions, efficient primer elongation is dependent on the matching nucleotide at the 3′ end (15). PCR conditions were optimized to enhance the inhibitory effect of a mismatch at this position. Specificity was considerably increased by using short annealing and elongation times (10 s) and a relatively high annealing temperature (66°C) (data not shown). Optimal conditions slightly differed for most primers. For instance, a high thermal stability (high GC content) at the 3′-terminal part of a primer reduced the effect of a mismatch at the 3′ end. To comply with the multiplex format, primer lengths and concentrations were adapted to a unique annealing temperature (66°C).

Fragment length and therefore the position of type-specific nucleotides within the HVR was critical to ensure proper size and type differentiation by agarose gel electrophoresis. Type-specific primers were thus designed for all active clades and genotypes after the positions and specificities of different candidate nucleotides were balanced and compatibility with experimental conditions (Table 1).

Although single point mutations within the primer binding sites should not affect the specificity of the reaction, the accumulation of mismatches may influence the efficiency of ampli-

TABLE 1. Sense primer combinations in the different multiplex PCR reactions

Mix and primer	Sequence	Concn (μM)	Position[a]
Clade mix[b]			
CIA	5′-GCAATGCATACTACTGAGGACAA-3′	0.4	1299–1321
CIB	5′-CAGGACAGTCGAAGGTCAGCC-3′	0.1	1563–1583
CIC	5′-CGAGATGGGGGGGTAAGGAAGATAT-3′	0.4	1396–1420
CID_a	5′-GATCAAAGTG*A*GA*A*TGAGCT*C*CCA-3′	0.2	1374–1397
CID_b	5′-GATCAAAGTG*A*GA*A*TGAGCT*A*CCA-3′	0.4	1374–1397
CID_c	5′-GATCAAAGTGGGAGTGAGCT*A*CCA-3′	0.2	1374–1397
CIG	5′-CCGGGCACAGCAGAGCAAA-3′	0.1	1468–1486
CIH	5′-CATTGACACTGCATCGGAGTA-3′	0.4	1529–1549
Genotype B Mix			
GrB3.1	5′-ACAGTCGAAGGTCAGCCGAT-3′	0.4	1567–1586
GrB3.2	5′-AGGACAGGAGGGTCAAACAGG-3′	0.4	1414–1434
Genotype D_I Mix			
GeD2	5′-GAGAAACCGGGTCCAGCAGAA-3′	0.2	1462–1482
GeD4	5′-CCCAGACAAGCCCAAGTGTCATTTA-3′	0.4	1341–1365
GeD6	5′-CCTAGACATTGACACTGCATCGGAGA-3′	0.4	1523–1548
GeD9	5′-GTCAAACAGAGTCGGGGAGAAGCA-3′	0.4	1425–1448
MVN	5′-CTGCAAGCCATGGCAGGAATC-3′	0.2	1599–1619
Genotype D_{II} Mix			
GeD3	5′-GCCCATCCTCCAACCAGCATG-3′	0.4	1500–1520
GeD5	5′-GGTATCACTGCCGAGGATGCG-3′	0.2	1260–1280
GeD7	5′-CCAAGATCTGCAGGACAGCCGAC-3′	0.2	1553–1575
GeD8	5′-GGGAGAAGCCAGGGAGAGCA-3′	0.4	1439–1458
MVN	5′-CTGCAAGCCATGGCAGGAATC-3′	0.4	1599–1619
Genotype G Mix			
GeG2	5′-GCAAATGATGCGAGAGCTGCTG-3′	0.4	1482–1503
GeG3	5′-CGGGATTGGGGGGTAAGGAAGATAAGAA-3′	0.4	1396–1423
Genotype H Mix			
GeH1	5′-CCAGGCAAGCCCAAGTCTCATTTT-3′	0.4	1342–1365
GeH2	5′-CTACAGAGAAACCGGGCTCAA-3′	0.4	1457–1477

[a] Nucleotide positions are given according to the assignments of Mori et al. (11).
[b] Degenerate positions for clade D primers are shown in italics.

fication depending on their relative positions. Some mutations were found relatively frequently within the clade D primer binding site. C1394 was present on 90% of all D5 strains, and all of the more recently detected D7 strains had two mutations in the primer region: G1384 and G1387. To compensate for this sequence variability, three similar primers (D_a, D_b, and D_c), with nucleotides matching known sequences at each of the three different sites, were combined in the cladetyping multiplex PCR (Table 1).

Cladetyping and genotyping. RT-PCR products of the complete HVR from strains of the different genotypes were first subjected to the cladetyping multiplex PCR containing type-specific sense and the common antisense primer revCG to identify the clade of the virus. Figure 3 shows that strains of each clade generated a single PCR product of the expected characteristic size, irrespective of the genotype to which the strain belonged within that clade. For instance, all D2-D9 genotypes gave a PCR product of the same length (277 bp), and no strain of another genotype gave a similar fragment. The fragment mix lanes show that PCR products of all clades can be clearly differentiated.

Depending on the clade identified, the corresponding genotyping multiplex PCR was used to assign the virus to its genotype (Fig. 3). Analysis of clade A and C strains was limited to the cladetyping reaction. Clade A consists of a single genotype, and only a single C genotype (C2) seemed to be active during the past 10 years (2). Two variants, B3.1 and B3.2, of the only active B genotype were distinguished by genotype B mix (Fig. 3). PCR products with a characteristic length were obtained for genotypes G2 and G3, as well as H1 and H2, with genotype G and genotype H mixes, respectively (Fig. 3). When all genotype D-specific primers were combined in a single multiplex PCR, the size resolution in the agarose gel was insufficient. Therefore, genotyping of clade D strains was split into two multiplex reactions: D_I containing primers GeD2, GeD4, GeD6, and GeD9 and D_{II} with the primers GeD3, GeD5, GeD7, and GeD8. All clade D templates were analyzed by both reactions. Thus, the specific primer of a given D genotype is present in only one of the two reaction mixtures. To obtain a single unequivocal band also in the irrelevant PCR mix, an additional sense primer (MVN), giving a 57-bp fragment with all genotypes, was included in both reaction mixtures D_I and D_{II} (Table 1). In some cases this fragment was also produced, together with the genotype-specific PCR product in the relevant mix (Fig. 3).

DISCUSSION

Type-specific nucleotides have been exploited to develop simple PCR-based assays to genotype viruses such as hepatitis B and C virus or Norwalk-like virus (8, 14, 16, 27). The robust-

mutation	A [36]	B3.1 [90]	B3.2 [80]	C2 [51]	D2 [6]	D3 [48]	D4 [48]	D5 [64]	D6 [66]	D7 [20]	D8 [12]	D9 [4]	G2 [7]	G3 [4]	H1 [58]	H2 [19]
Clade specific mutations																
A1321	97.2	1.1		2.0	16.7		2.1									
C1583		100.0	100.0													
T1420				100.0												
A1397					83.3	100.0	100.0	100.0	98.5	100.0	91.7	100.0				
A1486							2.1						100.0	100.0		
A1549															100.0	100.0
Genotype specific mutations																
T1586		100.0					2.1								3.4	
G1434			98.8			4.2	12.5			55.0						
A1482					100.0		6.3		1.5							
G1520						100.0										
A1365							100.0									
G1280			51.3					100.0								
A1548									100.0							
C1575										100.0						
A1458	2.8										100.0					68.4
A1448												100.0				
G1503													100.0			
A1423											8.3			100.0		
T1365															100.0	
A1477																100.0

FIG. 2. Frequency (percent) of type-specific nucleotides in the HVR of 613 MV strains. Shaded boxes correspond to the frequency of the type-specific nucleotides within the clade or genotype. Numbers of strains per genotype are indicated within brackets. Empty boxes correspond to 0%. Nucleotide positions are assigned according to the method of Mori et al. (11).

ness of these assays depends on a careful selection of suitable nucleotide differences between the different types. Although single nucleotide differences could be selected from any part of the viral genome, for most MV strains only the HVR of the nucleoprotein can be retrieved from sequence databases. Nucleotides that were found consistently in strains of a given clade or genotype, irrespective of time of isolation and geographic origin, are likely to be conserved also in the future. For most clades or genotypes a reliable number (19 to 268) of sequences was available, whereas the genotypes D2, D8, D9, G2, and G3 were represented with only few sequences (Fig. 2). The upper limit of sensitivity of the assay corresponds to the prevalence of selected type-specific nucleotides within their clades or genotypes. Among the 613 viruses analyzed here a total of 5 (0.8%) strains did not contain their type-specific nucleotide: clade A (one strain), genotype B3.2 (one strain), and clade D (three strains). The upper limit of specificity depends on the frequency of a type-specific nucleotide to occur also in another clade or genotype. This was the case for nine (1.4%) strains, corresponding to a theoretical specificity of 98.6%. The resulting positive and negative predictive values of the assay are 99.2 and 98.6%, respectively, when the results of both cladetyping and genotyping PCR are considered (Fig. 2). Strains giving no or several bands, due to missing type-specific nucleotides or additional nucleotides specific for a different type, must be sequenced for further characterization.

Normally MV genotyping relies on RT-PCR, followed by a nested PCR, which generates the template for the sequencing reaction. Here, the sequencing reaction is replaced by two consecutive multiplex PCRs: one to identify the clade, followed by a second one to identify the corresponding genotype. PCR fragments are analyzed by conventional agarose gel electrophoresis and visual comparison of fragment size with standards, replacing complex phylogenetic analyses. Theoretically, the cladetyping and all five genotyping multiplex PCRs could be performed in parallel, since the instrumental conditions are identical. However, in this case only the genotyping results corresponding to the identified clade must be considered, since among the known sequences some genotype-specific nucleotides are also found in strains belonging to a different clade (Table 1).

Mutations in the primer region could potentially affect the efficiency of the PCR amplification and may lead to false-negative results. Therefore, sequence variability within the complete primer region was carefully analyzed. Three homologous D-specific primers (D_a, D_b, and D_c; see Table 1) were included in the clade mix to compensate for sequence variability in the clade D primer region. For the remaining types, only little variability was found in the primer region. Only single mutations were found in the different primer binding sites, except in 4 B3 strains (two mutations). If all mutations in the primer binding region would affect the assay the specificity would be unaffected but the theoretical sensitivity would be reduced to 86.4%. However, only mutations located in the binding site of the 3′-terminal primer part impaired the formation of the corresponding PCR product under the experimental conditions recommended here. Such a mutation may interfere with both primer annealing and extension. Figure 4 shows that, among all mutations located in the primer binding regions, only a small number is found in the binding site of the 3′-terminal primer part.

Three or more different strains were tested for most genotypes. Although of the most recently identified genotypes D9,

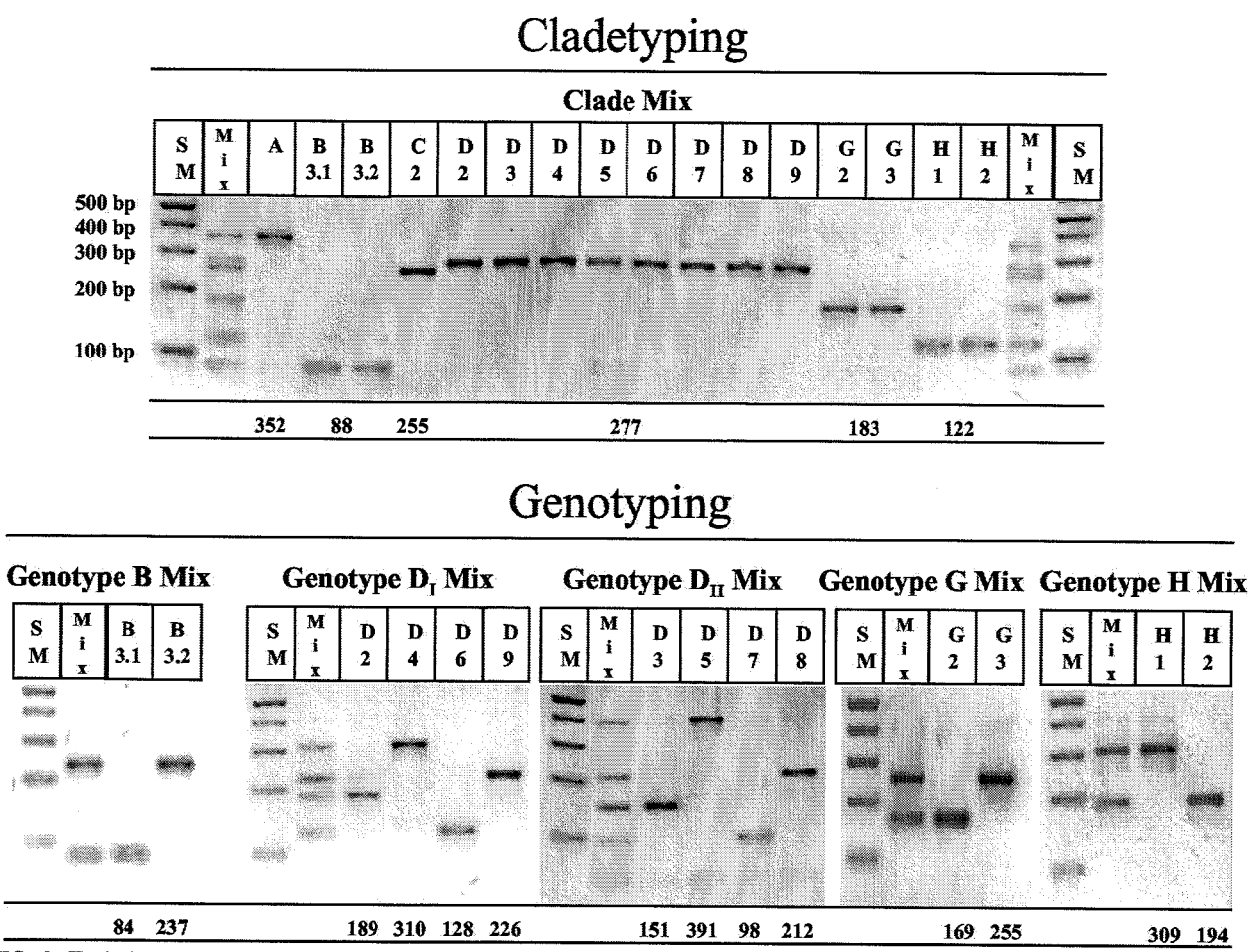

FIG. 3. Typical agarose gel electrophoresis pattern of PCR products from different MV clades and genotypes as determined by multiplex PCR. Numbers under the lanes indicate the fragment sizes in base pairs. Lanes with multiple bands correspond to fragment mix standard (Mix). SM, size marker.

G2, G3, and H2 only a single strain was available for testing, all other published sequences of these genotypes showed no mutation within the primer binding region that could interfere with amplification.

During the past 5 years a number of new genotypes have been identified, and more identifications may follow (10). Genotypes that are considered inactive and that were therefore not included in the present study (B1, B2, C1, D1, E, and F) may resurface in areas with little molecular epidemiological surveillance. For instance, clade G was considered inactive before it was detected in Indonesia in 1997, 14 years after its last detection in Berkeley, Calif. (3, 6, 19). As new genotypes are described, additional specific primers will have to be included in the multiplex PCR. Obviously, only known genotypes can be identified with the present method.

The present simplified genotyping approach is based on techniques that are commonly used in many diagnostic laboratories throughout the world. This method brings MV genotyping within reach of many more laboratories, in developing countries in particular, although the practicability in these countries needs to be confirmed. The protocol (including reagents) described here must be strictly applied by experienced lab workers to obtain reliable results. The assay is highly specific and can be adapted to different levels of characterization, and new genotypes and mutations of particular interest can be added with relative ease. However, ultimate confirmation of the results can only be obtained from sequence analysis.

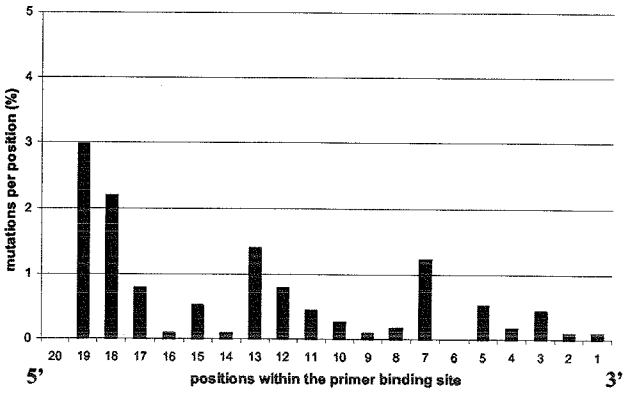

FIG. 4. Cumulated sequence variability in the different positions of the corresponding binding sites of all primers from 5′ to 3′. The data represent all mutations resulting from the comparison of all published strain sequences to their clade- and/or genotype-specific primer. Position 0 corresponds to the 3′-terminal nucleotide of the primer.

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REFERENCES

1. **Baczko, K., I. Pardowitz, B. K. Rima, and V. ter Meulen.** 1992. Constant and variable regions of measles virus proteins encoded by the nucleocapsid and phosphoprotein genes derived from lytic and persistent viruses. Virology **190:**469–474.
2. **Barrero, P. R., R. O. Zandomeni, and A. S. Mistchenko.** 2001. Measles virus circulation in Argentina: 1991–1999. Arch. Virol. **146:**815–823.
3. **Bellini, W. J., and P. A. Rota.** 1998. Genetic diversity of wild-type measles viruses: implications for global measles elimination programs. Emerg. Infect. Dis. **4:**29–35.
4. **Chibo, D., M. Riddell, M. Catton, and C. Birch.** 2002. Novel measles virus genotype, East Timor and Australia. Emerg. Infect. Dis. **8:**735–737.
5. **Chibo, D., M. Riddell, M. Catton, M. Lyon, G. Lum, and C. Birch.** 2003. Studies of measles viruses circulating in Australia between 1999 and 2001 reveals a new genotype. Virus Res. **91:**213–221.
6. **de Swart, R. L., P. M. Wertheim-van Dillen, R. S. van Binnendijk, C. P. Muller, J. Frenkel, and A. D. Osterhaus.** 2000. Measles in a Dutch hospital introduced by an immuno-compromised infant from Indonesia infected with a new virus genotype. Lancet **355:**201–202.
7. **Hanses, F., A. T. Truong, W. Ammerlaan, O. Ikusika, F. Adu, A. O. Oyefolu, S. A. Omilabu, and C. P. Muller.** 1999. Molecular epidemiology of Nigerian and Ghanaian measles virus isolates reveals a genotype circulating widely in western and central Africa. J. Gen. Virol. **80:**871–877.
8. **Kirschberg, O., C. Schuttler, R. Repp, and S. Schaefer.** 2004. A multiplex-PCR to identify hepatitis B virus-genotypes A-F. J. Clin. Virol. **29:**39–43.
9. **Kreis, S., E. Vardas, and T. Whistler.** 1997. Sequence analysis of the nucleocapsid gene of measles virus isolates from South Africa identifies a new genotype. J. Gen. Virol. **78:**1581–1587.
10. **Kremer, J. R., and C. P. Muller.** 2003. Molecular epidemiology of measles in Asia. Curr. Top. Virol. **3:**165–181.
11. **Mori, T., K. Sasaki, H. Hashimoto, and S. Makino.** 1993. Molecular cloning and complete nucleotide sequence of genomic RNA of the AIK-C strain of attenuated measles virus. Virus Genes. **7:**67–81.
12. **Mulders, M. N., and C. P. Muller.** 2002. Molecular epidemiology of measles virus, p. 237–272. *In* Thomas Leitner (ed.), The molecular epidemiology of human viruses. Kluwer Academic Publishers, Boston, Mass.
13. **Mulders, M. N., A. T. Truong, and C. P. Muller.** 2001. Monitoring of measles elimination using molecular epidemiology. Vaccine **19:**2245–2249.
14. **Naito, H., S. Hayashi, and K. Abe.** 2001. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. J. Clin. Microbiol. **39:**362–364.
15. **Newton, C. R., A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith, and A. F. Markham.** 1989. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). Nucleic Acids Res. **17:**2503–2516.
16. **Ohno, O., M. Mizokami, R. R. Wu, M. G. Saleh, K. Ohba, E. Orito, M. Mukaide, R. Williams, and J. Y. Lau.** 1997. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J. Clin. Microbiol. **35:**201–207.
17. **Rota, J. S., J. L. Heath, P. A. Rota, G. E. King, M. L. Celma, J. Carabana, R. Fernandez-Munoz, D. Brown, L. Jin, and W. J. Bellini.** 1996. Molecular epidemiology of measles virus: identification of pathways of transmission and implications for measles elimination. J. Infect. Dis. **173:**32–37.
18. **Rota, P. A., A. E. Bloom, J. A. Vanchiere, and W. J. Bellini.** 1994. Evolution of the nucleoprotein and matrix genes of wild-type strains of measles virus isolated from recent epidemics. Virology **198:**724–730.
19. **Rota, P. A., S. Liffick, S. Rosenthal, B. Heriyanto, and K. B. Chua.** 2000. Measles genotype G2 in Indonesia and Malaysia. Lancet **355:**1557–1558.
20. **Samuel, D., S. Beard, H. Yang, N. Saunders, and L. Jin.** 2003. Genotyping of measles and mumps virus strains using amplification refractory mutation system analysis combined with enzyme immunoassay: a simple method for outbreak investigations. J. Med. Virol. **69:**279–285.
21. **Santibanez, S., A. Tischer, A. Heider, A. Siedler, and H. Hengel.** 2002. Rapid replacement of endemic measles virus genotypes. J. Gen. Virol. **83:**2699–2708.
22. **Takahashi, M., T. Nakayama, Y. Kashiwagi, T. Takami, S. Sonoda, T. Yamanaka, H. Ochiai, T. Ihara, and T. Tajima.** 2000. Single genotype of measles virus is dominant, whereas several genotypes of mumps virus are co-circulating. J. Med. Virol. **62:**278–285.
23. **Truong, A. T., S. Kreis, W. Ammerlaan, H. K. Hartter, F. Adu, S. A. Omilabu, A. O. Oyefolu, G. A. Berbers, and C. P. Muller.** 1999. Genotypic and antigenic characterization of hemagglutinin proteins of African measles virus isolates. Virus Res. **62:**89–95.
24. **Truong, A. T., M. N. Mulders, D. C. Gautam, W. Ammerlaan, R. L. de Swart, C. C. King, A. D. Osterhaus, and C. P. Muller.** 2001. Genetic analysis of Asian measles virus strains: new endemic genotype in Nepal. Virus Res. **76:**71–78.
25. **World Health Organization.** 2003. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference strains. Wkly. Epidemiol. Rec. **78:**229–232.
26. **Xu, W., A. Tamin, J. S. Rota, L. Zhang, W. J. Bellini, and P. A. Rota.** 1998. New genetic group of measles virus isolated in the People's Republic of China. Virus Res. **54:**147–156.
27. **Yuen, L. K., M. G. Catton, B. J. Cox, P. J. Wright, and J. A. Marshall.** 2001. Heminested multiplex reverse transcription-PCR for detection and differentiation of Norwalk-like virus genogroups 1 and 2 in fecal samples. J. Clin. Microbiol. **39:**2690–2694.

XI. Génotypage de souches sauvages du virus de la rougeole circulant en Russie et au Vietnam par PCR multiplex

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Résumé de l'article

Afin d'évaluer la performance de la méthode, 59 souches sauvages non-caractérisées du virus de la rougeole ont été soumises au génotypage par PCR multiplex. Les souches sauvages ont été obtenues entre 2000 et 2006 à partir d'échantillons cliniques prélevés sur des patients de 12 régions différentes de la Russie (n=46) ainsi que de deux villes (Hanoi, Nha Trang) du Vietnam (n=13) en 2003. Alors que la méthode décrite auparavant avait été établie en utilisant des isolats connus, il s'agissait maintenant de valider et d'optimiser la procédure sur des souches non-caractérisées qui circulaient dans différentes régions du monde. De plus la partie C-terminale du gène N à été séquencée pour toutes les souches analysées, afin de comparer les résultats obtenus par PCR multiplex à la procédure de génotypage standard.
Bien que le clade et le génotype correct aient pu être déterminés pour la majorité des virus (48/59; 81,4%) par multiplex PCR, certaines souches ont donné des résultats ambigus. Sept souches du Vietnam, identifiées comme génotype H1 par séquençage, ont donné un mélange de différents fragments PCR dans la réaction spécifique des clades. D'autre part quatre souches D6 de Russie n'ont pas donné de fragment PCR dans la réaction spécifique des génotypes D. Dans les deux cas, la présence simultanée de deux mutations par rapport aux amorces spécifiques du clade H et du génotype D6 a été détectée par séquençage. L'utilisation de nouvelles amorces, portant une des deux mutations décrites a permis d'obtenir les fragments caractéristiques du clade H et du génotype D6 respectivement avec toutes les souches testées.
La majorité des souches russes appartenaient au génotype D6 (n=40). Les autres ont été identifiées comme génotype D4 (n=4) et H1 (n=2). Au Vietnam toutes les souches (n=6) provenant de Hanoi appartenaient au génotype H2. Parmi ces dernières, cinq souches avaient des séquences identiques par rapport à des isolats obtenus dans la même région en 1998, ce qui indique que ces virus n'ont cessé de circuler pendant au moins cinq ans dans la région. Les souches obtenues à Nha Trang (n=7) appartenaient au génotype H1. Puisque les souches appartenant au clade H sont dominantes aussi dans d'autres pays d'Asie – par exemple en Chine - une procédure simplifiée de la

méthode a été établie: elle permet de distinguer directement H1, H2 et non H1/nonH2.

Cette étude a donc montré que la méthode multiplex PCR permet de génotyper la plus grande partie des souches analysées, et que l'adaptation de la méthode à de nouveaux variants ainsi qu'à des situations particulières est relativement facile.

Genotyping of recent Measles Virus strains from Russia and Vietnam by nucleotide specific multiplex PCR

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Abstract

The nucleoprotein genes of 49 measles virus strains circulating in Russia between 2000 and 2006 and in Vietnam in 2003 were analysed by genotype-specific PCR and the results were compared with their sequences. The sequences revealed the presence of genotypes H1 and H2 in the Centre (Nha Trang) and the North (Hanoi) of Vietnam, respectively. The relative diversity of the H2 strains suggested an endemic circulation of these viruses in the capital. In contrast genotype H1 strains from Nha Trang were genetically homogenous, which may indicate a recent importation. The strains obtained from 12 different regions of the Russian Federation were assigned to the genotypes H1, D4 and D6. Most strains (81.4%) were correctly genotyped by a multiplex PCR method, described earlier by us, which was sensitive to genotype specific mutations. Ambiguous or negative results for some clade H and genotype D6 strains were due to point mutations in the type-specific primer binding sites. After exchanging a single nucleotide in both the clade H and the genotype D6 specific primers, all strains were correctly assigned to their genotype. A simplified procedure for use in Vietnam was developed to distinguish directly between genotypes H1 and H2 and any non-H genotype. These results demonstrate that our multiplex PCR method can be easily adapted to new sequence variants or specific epidemiological situations, and thus be very useful for rapid genotyping of large numbers of samples even in laboratories which do not have sequencing facilities.

Introduction

Integration of routine measles vaccination into the Expanded Programme of Immunization (EPI) has dramatically reduced measles morbidity and mortality worldwide (6). In the Americas and a number of other countries, elimination of indigenous measles virus (MV) strains was demonstrated by molecular epidemiology (2, 12). Molecular epidemiology has proven useful to distinguish between endemic and imported MV strains; therefore genotyping becomes increasingly important as countries move towards measles elimination (5, 12, 16). Nevertheless, measles is still endemic in many regions in Africa and Asia, where reinforced vaccination strategies are critical for further progress of measles control (3, 4, 9, 15).

Routine measles vaccination was introduced in Vietnam during the 1990's and was invigorated in 2002/2003 by a nationwide catch-up campaign (1). Annual numbers of reported cases continued to decrease from more than 16000 in 2000 to less than 300 by 2004 (20). At this stage measles strain characterisation becomes important to demonstrate the success of the vaccination strategy and progress towards elimination of indigenous strains. In Russia genotype D4 strains were circulating between 1999 and 2003. Since 2003 very similar D6 strains were identified in different outbreaks throughout the Russian Federation, suggesting continuous chains of transmission of these viruses (Shulga et al., in preparation).

Both in Vietnam and Russia, monitoring of virus strains would allow to distinguish between multiple reintroduction events or the continuous circulation of similar strains. The latter would suggest, that numbers of susceptibles are sufficient to support the uninterrupted circulation of the virus,

whereas pseudo-outbreaks caused by reintroduced viruses indicate that transmission is interrupted for lack of susceptibles.

We have recently developed a multiplex PCR method to identify MV clades and genotypes without sequencing (8). The method is based on single type-specific mutations in the hypervariable region (HVR, 450 C-terminal nucleotides) of the N-gene. The corresponding nucleotides were included as 3'-terminal base into type- specific primers, giving PCR fragments of characteristic lengths for each clade or genotype. In a first step, uncharacterized strains are analyzed by a mix of clade specific primers. When the clade is identified, they are further analyzed by a second multiplex PCR to determine the genotype, using genotype-specific primers of the corresponding clade. Because of the complexity of clade D, two independent multiplex PCRs (genotype mix D_I and D_{II}), each including a different set of specific primers are used to subtype clade D strains. The method distinguishes between clades (A, B, C, D, G, H) and genotypes (A, B3.1-B3.2, C2, D2-D9, G2-G3, H1-H2) without the need for sequencing (8). Here we evaluated the performance of the non-sequencing genotyping method using clinical specimens and uncharacterized virus isolates obtained from patients in Russia and Vietnam and adapt it to the specific epidemiological situation of Vietnam.

Materials and Methods

Clinical specimens and MV isolates

Clinical specimens from 36 measles patients were collected between 2000 and 2006 in 12 different regions of the Russian Federation: Astrakhan (n=2),

Dagestan (n=1), Irkutsk (n=1), Novokuznezk (n=3), Krasnoyarsk (n=6), Kursk (n=2), Saransk (n=1), Novosibirsk (n=1), Moscow (n=8), Rostov (n=7), Ryazan (n=1), Vladimir (n=3). Samples used for RNA extraction included 22 throat swabs, 12 urines, 2 PBMCs and 10 MV culture supernatants. Culture supernatants from measles strains collected in the north (Hanoi, n=6) and the center (Nha Trang, n=7) of Vietnam between February and May 2003 were also included. Virus isolates were obtained by co-cultivation of cellular fractions from different clinical specimens with Vero (Vietnam), Vero Slam or B95a cells (Russia).

RNA isolation and RT-PCR

Total RNA was extracted from 140 µl of cell culture supernatant, or cellular fractions of urine, throat swabs and PBMCs in virus transport medium using QIAamp Viral RNA kit (Qiagen, Hilden, D). Specific cDNA of MV nucleoprotein was synthesized by reverse transcription, using SuperscriptIII Reverse Transcriptase (Invitrogen, Merelbeke, B) and primer MN5 (nt 1113-1134, 5'-GCCATGGGAGTAGGAGTGGAAC, (17)). Measles virus cDNA was amplified by nested PCR using primers MN5 and MN6 (nt 1773-1754, 5'-CTGGCGGCTGTGTGGACCTG, (17)) for the first round and primers Nf1a (nt 1199-1224, 5'-CGGGCAAGAGATGGTAAGGAGGTCAG) and Nr7a (nt 1725-1703, 5'-AGGGTAGGCGGATGTTGTTCTGG) for the second round. Both PCR reactions were done in a total volume of 25 µl reaction mix including 1.8 mM $MgCl_2$, 1x PCR buffer, 0.2 mM dNTPs, 0.5 U Platinum Taq (Invitrogen, Merelbeke, B) and 0.8 µM forward and reverse primer (Eurogentec, Seraing, B). 1 µl of cDNA or 5 µl of 1st round product (50x diluted in H_2O) were added

as template. Cycling conditions were as follows: initial denaturation at 94°C for 2 min, 35 (1st round) or 30 (2nd round) cycles of amplification at 94°C for 30 s, 55°C (1st round) or 58°C (2nd round) for 1 min and 72 °C for 1 min, and a final extension at 72°C for 5 min.

Clade- and Genotype-specific PCR

All samples were also submitted to clade- and genotype specific multiplex PCRs essentially as described before (8). Several minor modifications were performed to identify new variants of MV strains and further optimize the method: (i) 5 µl of MN5-MN6 PCR product (50x diluted in H_2O) were used as template instead of nested PCR fragments; (ii) a new clade H specific primer (ClH_{10C}, Table 1) and a new genotype D6 specific primer ($GeD6_{15A}$, Table 1) were included to the clade or genotype D_I mix, to compensate for sequence variability among recent clade H and D6 strains. Reagents and cycling conditions were as described before (8).

Clade H templates were also submitted to an additional multiplex PCR including primers GeH1 (nt 1342–1365, 5'-CCAGGCAAGCCCAAGTCTCATTTT), GeH2 (nt 1457–1477, 5'-CTACAGAGAAACCGGGCTCAA), Nf1a and Nr7a. This PCR was done in a total volume of 25 µl containing 1.7 mM $MgCl_2$, 1x PCR buffer, 0.2 mM dNTP, 1.0 U of Platinum Taq DNA polymerase (Invitrogen, Merelbeke, B) and primers: GeH1 (0.4 µM), GeH2 (0.4µM), Nf1 (0.4µM) and Nr7 (0.8µM) (Eurogentec, Seraing, B). PCR conditions were identical to the clade-and

genotype specific multiplex PCRs described before (2 min 95° C; 25x 30s 95°C, 10s 66 °C, 10s at 72° C; 5 min 72°C).

All PCRs were performed on a Mastercycler Gradient (Eppendorf, Hamburg, D) and the size of PCR products was determined by agarose gel electrophoresis (Invitrogen, Merelbeke, B).

Cycle Sequencing

Nf1-Nr7 nested PCR products were purified by Jetquick PCR product Purification Spin kit (Genomed, Lohne, D). 25 cycles of cycle sequencing (2 min elongation) were performed using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Nieuwerkerk, NL) with Nf1a or Nr7a primers (0.5 µM) and 10 ng of purified PCR product. Cycle sequencing products were analysed on an ABI 3130 Genetic Analyser (Applied Biosystems, Nieuwerkerk, NL). Sequences were aligned using ClustalW and phylogenetic tree was constructed by neighbour-joining method (Kimura 2-parameter) using the MEGA (version 3.0) software. All new sequences were entered into GenBank under the accession numbers AM087620-AM087632.

Results

Sequencing of MV strains from the Russian Federation and Vietnam

The complete HVR of all strains from Russia and Vietnam was sequenced after partial genomic amplification by RT-PCR. In Russia, two different clades

and three different genotypes were identified by phylogenetic analysis: genotype D6 (n=30) and D4 (n=4) and genotype H1 (n=2). Four and seven variants, differing by a maximum of 4 and 5 nucleotides, were identified among genotype D4 and D6 strains, whereas the two H1 sequences were identical (Fig.1). Sequence analysis of the virus isolates from Vietnam revealed two different genotypes, H1 (n=7) and H2 (n=6), including two and four different variants differing by a maximum of 1 and 6 nucleotides, respectively (Fig. 1). Sequences obtained from the same patient but different clinical materials were always identical.

Cladetyping by Multiplex PCR

The above strains were also used to evaluate and further optimize our previously described non-sequencing genotyping assay. Clade- and genotyping by multiplex PCR was performed under double-blind conditions directly on the clinical material whenever available or virus isolates. All variants circulating in Russia were correctly assigned to either clade D or clade H by cladetyping multiplex PCR (results not shown). Also genotype H2 strains from Vietnam gave a distinct fragment with the typical size of clade H. In contrast, genotype H1 strains gave a mix of clade H (122 bp) and clade D (277 bp) specific fragments in the cladetyping PCR (Fig. 2). Sequence analysis revealed that these strains had two point mutations (T1538C and G1544A) in the clade H primer (ClH) binding site (Table 1). Thus, the efficiency of clade H specific PCR was significantly reduced, which permitted another clade-specific fragment to come-up, although only the clade H

specific mutation was present in the sequence. After including one of the two mutations (T1538C) into the clade H specific primer (ClH_{10C}, Table 1) both genotype H1 and genotype H2 strains from Vietnam gave a single PCR product with a size characteristic of clade H (Fig.2).

Genotyping by Multiplex PCR

Clade D strains from Russia were further analysed using the two genotype D specific multiplex PCRs (genotype D_I and D_{II} mix). All D4 strains (n=4) gave the D4 specific PCR product in the genotype D_I mix and no genotype specific fragment in the D_{II} mix (Fig. 3). On the other hand only 5 of 8 genotype D6 variants could be assigned to the correct genotype on the basis of the genotype specific PCR (Fig. 3). The other three variants had two point mutations (C1538A, and either C1529T or T1532C) in the genotype D6 primer binding site (Table 1) and gave no or only weak genotype specific bands in both genotype D specific multiplex PCRs (Fig. 3). Sequence analysis revealed, that the mutation C1538A was present in all D6 strains from Russia. However, only those with an additional mutation (either C1529T or T1532C) did not give a D6 fragment. Therefore we have modified the D6 primer GeD6 to include the mutation C1538A ($GeD6_{A15}$, Table 1). Using this primer all D6 variants gave the characteristic D6-specific PCR fragment (Fig. 3), although the band was still weak for MVi/Dagestan.RUS/20.03 .

All clade H strains were submitted to genotype H specific PCR after cladetyping. In all cases, both H1 and H2 strains were correctly assigned to

their genotype by virtue of the characteristic H1- (309 bp) or H2- (194 bp) specific PCR products (Fig. 4).

Genotype H1, H2 specific PCR

Considering the persistent circulation of clade H strains in South-East Asia (7, 9, 10, 19) a one step genotyping assay for H1 and H2 could be of major interest. Therefore we have designed a multiplex PCR reaction to specifically distinguish between genotypes H1, H2 and non-H genotypes in a single multiplex PCR. The reaction mix contains genotype H1 and genotype H2 specific forward primers, as well as the forward (Nf1) and reverse (Nr7) primer of our diagnostic nested PCR. With this mix H1 or H2 specific DNA fragments (261 bp or 384 bp) were produced if the template DNA belonged to either one of the clade H genotypes (Fig. 5). On the other hand only the Nf1-Nr7 PCR product (526 bp) was produced with viruses of any other genotype. The examples of a clade A and a clade B strain are shown in Fig. 5.

Discussion

WHO considers molecular characterization and genotyping as a main component of measles surveillance and control (19). Although sequencing will always be the gold standard, we had developed a rapid and less time consuming method, which brings genotyping within reach of many laboratories, which do not have sequencing facilities, and facilitates screening of large numbers of samples. The technique was developed using virus isolates of most known genotypes.

Here we have evaluated the performance of the non-sequencing genotyping method, using clinical specimens and uncharacterized virus isolates obtained from measles patients in Vietnam and Russia between 2000 and 2006. Measles RNA was reverse-transcribed and amplified by a single round PCR, before being characterized by clade- or genotype-specific multiplex PCRs. Although most strains (48/59 strains; 81,4%) or sequence variants (14/19 variants; 73,7%) were correctly genotyped, some strains gave ambiguous results in either the clade- or the genotype specific multiplex PCR. An ambigous mix of clade H and clade D fragments was obtained for all H1 strains from Nha Trang. No genotype specific fragment was obtained for three D6 variants from Russia. In both cases, two point mutations were identified in the binding site of the corresponding specific primers, which impaired the PCR amplification of the correct fragment as discussed before (8). In both cases this was corrected by including one of the two mutations into the corresponding primer. A single mismatch did not reduce the PCR efficiency to compromize genotyping. Among all published strains (Genbank, August 2005) only 23 of 918 (2.5%) had more than one point mutation in the binding site of their clade or genotype specific primer. Moreover the present examples show that primer sequences can be easily optimized as new variants become dominant in a region.

In a particular region with continued measles virus transmission a non-sequencing genotyping assay may be particularly helpful, to demonstrate the persistence of certain genotypes. The simplified multiplex PCR assay which distinguishes directly between genotypes H1 and H2 and any non-H genotype serves this purpose for measles surveillance in Vietnam and other Asian

countries, were clade H strains dominate (9, 19). In case no H1 or H2 fragment is seen, the PCR fragment obtained with all non-H genotypes comprizes the complete HVR and can be directly used for sequencing.

Two different genotypes, H1 and H2, were detected among strains from Vietnam in 2003 (Fig.1). All strains (n=6) obtained from measles patients in Hanoi (North of Vietnam) were of the H2 genotype, whereas the strains (n=7) collected during the same period in Nha Trang (Center of Vietnam) belonged to genotype H1. Among the H2 strains, 4 variants were identified, which differed by 1 to 8 nucleotides (0.2 to 1.8 %). On the basis of sequence diversity between MV strains isolated in 1998, Liffick at al. (11) proposed that H2 was the indigenous genotype in Vietnam. We found that three out of four of H2 variants from 1998 were still present in Hanoi in 2003 (Figure 6). This suggests (i) that continous chains of MV transmission must have persisted for at least 5 years and (ii) that measles strains can circulate for several years without mutating. Endemic transmission of genotype H2 has not been described anywhere else outside of Vietnam. Some H2 strains were found as sporadic imported cases in the United States, at least one of which (MVi/Minnesota.USA/13.97, Fig. 6) was epidemiologically linked to Vietnam (16). An additional H2 sequence from Taiwan (Taoyuan.TWN/24.94/2) (AY737405) identical to a 1998/2003 Hanoi strain could be retrieved from GenBank (AY737405).

Only 2 variants with one nucleotide difference were found among the seven H1 strains. Genotype H1 had not been described in Vietnam before. The limited diversity of these H1 strains suggests a limited outbreak in Na Trang caused by an imported virus. Genotype H1 has been reported from many

other countries in South-East Asia including China, Japan, Republic of Korea and Mongolia (11, 13, 14). The H1 strains from Nha Trang 2003 were genetically most closely related to a variant detected in Taiwan (MVs/Taipei.TWN/36.97) and an isolate (MVi/Massachusetts.USA/2.98) imported into the United States from China (16) (Fig. 6). Thus both the genotype of the Vietnam strains and their genetic diversity suggest that the virus continued to be endemic in Hanoi after 1998, whereas in Nha Trang, the outbreak was caused by limited circulation of an imported strain. MV genotype distribution in Russia has been discussed elsewhere (Shulga et al. in preparation).

The above sequencing results show that periodic genotyping of measles virus is important to distinguish between continued transmissions and virus reintroduction. We showed here, that our multiplex PCR based method allows genotype identification of most MV strains obtained directly from a variety of clinical samples in two different geographical settings. The method can be easily adapted to new sequence variants and a simplified version of the technique can be tailored to the specific needs of a country or region such as shown here for Vietnam.

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References

1. **Ali, M., G. D. Canh, J. D. Clemens, J. K. Park, L. von Seidlein, T. T. Minh, D. V. Thiem, H. L. Tho, and D. D. Trach.** 2005. The use of a computerized database to monitor vaccine safety in Viet Nam. Bull World Health Organ **83:**604-10.
2. **CDC.** 2004. Progress toward measles elimination--region of the Americas, 2002-2003. MMWR Morb Mortal Wkly Rep **53:**304-6.
3. **CDC.** 2004. Progress towards sustainable measles mortality reduction--South-East Asia region, 1999-2002. Wkly Epidemiol Rec **79:**241-4.
4. **CDC.** 2003. Update: global measles control and mortality reduction--worldwide, 1991-2001. MMWR Morb Mortal Wkly Rep **52:**471-5.
5. **Hanses, F., R. van Binnendijk, W. Ammerlaan, A. T. Truong, L. de Rond, F. Schneider, and C. P. Muller.** 2000. Genetic variability of measles viruses circulating in the Benelux. Arch Virol **145:**541-51.
6. **Henao-Restrepo, A. M., P. Strebel, E. John Hoekstra, M. Birmingham, and J. Bilous.** 2003. Experience in global measles control, 1990-2001. J Infect Dis **187 Suppl 1:**S15-21.

7. **Jin, L., Y. J. Sun, L. Ge, and D. W. Brown.** 1998. Characterization of a new genotype of measles virus detected in China and England. Epidemiol Infect **121**:691-7.

8. **Kremer, J. R., F. Fack, C. M. Olinger, M. N. Mulders, and C. P. Muller.** 2004. Measles virus genotyping by nucleotide-specific multiplex PCR. J Clin Microbiol **42**:3017-22.

9. **Kremer, R. J., and C. P. Muller.** 2003. Molecular epidemiology of measles in Asia. Current Topics in Virology **3**:165-182.

10. **Liffick, S. L., N. Thi Thoung, W. Xu, Y. Li, H. Phoung Lien, W. J. Bellini, and P. A. Rota.** 2001. Genetic characterization of contemporary wild-type measles viruses from Vietnam and the People's Republic of China: identification of two genotypes within clade H. Virus Res **77**:81-7.

11. **Liffick, S. L., N. Thi Thoung, W. Xu, Y. Li, H. Phoung Lien, W. J. Bellini, and P. A. Rota.** 2001. Genetic characterization of contemporary wild-type measles viruses from Vietnam and the People's Republic of China: identification of two genotypes within clade H. Virus Res **77**:81-7.

12. **Muller, C. P., and M. N. Mulders.** 2002. Molecular epidemiology of measles virus, p. 237-272. *In* T. Leitner (ed.), The Molecular Epidemiology of Human Viruses. Kluwer Academic Publishers, Boston.

13. **Na, B. K., J. M. Shin, J. Y. Lee, G. C. Shin, Y. Y. Kim, J. S. Lee, J. K. Lee, H. W. Cho, H. J. Lee, P. A. Rota, W. J. Bellini, W. J. Kim, and C. Kang.** 2003. Genetic and antigenic characterization of measles

viruses that circulated in Korea during the 2000-2001 epidemic. J Med Virol **70:**649-54.

14. **Nakayama, T., M. Fujino, and N. Yoshida.** 2004. Molecular epidemiology of measles virus in Japan. Pediatr Int **46:**214-23.

15. **Otten, M., R. Kezaala, A. Fall, B. Masresha, R. Martin, L. Cairns, R. Eggers, R. Biellik, M. Grabowsky, P. Strebel, J. M. Okwo-Bele, and D. Nshimirimana.** 2005. Public-health impact of accelerated measles control in the WHO African Region 2000-03. Lancet **366:**832-9.

16. **Rota, P. A., S. L. Liffick, J. S. Rota, R. S. Katz, S. Redd, M. Papania, and W. J. Bellini.** 2002. Molecular epidemiology of measles viruses in the United States, 1997-2001. Emerg Infect Dis **8:**902-8.

17. **Santibanez, S., A. Tischer, A. Heider, A. Siedler, and H. Hengel.** 2002. Rapid replacement of endemic measles virus genotypes. J Gen Virol **83:**2699-2708.

18. **Shulga, S., M. Naumova, T. Mamaeva, L. Nesterina, A. Gerasimova, O. Cvirkun, P. Rota, W. Bellini, C. Muller, J. Kremer, and N. Tikhonova.** 2006. Molecular epidemiology of wild-type measles virus in Russian Federation. in preparation.

19. **WHO - World Health Organization.** 2005. Global measles and rubella laboratory network--update. Wkly Epidemiol Rec **80:**384-8.

20. **WHO - World Health Organization.** http://www.who.int/immunization_monitoring/en/globalsummary/timeseries/tsincidencemea.htm.

Table 1: Nucleotide sequences of genotype H1 and genotype H2 strains from Vietnam in the binding site of the clade H specific primer (CIH, nt 1529-1549) and nucleotide sequences of the different genotype D6 variants from Russia in the binding site of the genotype D6 specific primer (GeD6, nt 1523-1548).

Clade H	**Sequences**
Primer CIH	5'-CATTGACACTGCATCGGAGTA-3'
H1 strains	5'-CATTGACAC**C**GCATC**A**GAGTA-3'
H2 strains	5'-CATTGACACTGCATCGGAGTA-3'
Primer CIH_{10C}	5'-CATTGACAC**C**GCATCGGAGTA-3'

Genotype D6	**Sequences**
Primer GeD6	5'-CCTAGACATTGACACTGCATCGGAGA-3'
D6 variant 1	5'-CCTAGACATTGACA**A**TGCATCGGAGA-3'
D6 variant 2	5'-CCTAGACAT**C**GACA**A**TGCATCGGAGA-3'
D6 variant 3	5'-CCTAGA**T**ATTGACA**A**TGCATCGGAGA-3'
Primer $GeD6_{15A}$	5'-CCTAGACATTGACA**A**TGCATCGGAGA-3'

Legends to Figures:

Figure 1: Genotyping of measles virus strains from Russia and Vietnam by sequencing and phylogenetic analysis. The phylogenetic tree was based on the 450 C-terminal nucleotides of the N gene and includes all sequences from Russia and Vietnam as well as WHO reference sequences of all genotypes. Mutations in the genotype D6 and the clade H specific primer binding sites are indicated for those strains which were not detected with the corresponding multiplex PCRs.

Figure 2: Cladetyping PCR of all measles virus strains from Vietnam. Results obtained with ClH (top gel) or ClH_{10C} (bottom gel) primer in the cladetyping primer mix are shown.

Figure 3:

Genotyping multiplex PCR with genotype D_I mix of all clade D variants from Russia. D_I: Mix of genotype D2 (189 bp), D4 (310 bp), D6 (128 bp) and D9 (226 bp) specific fragments. Results obtained for representative strains using GeD6 (top gel) as well as $GeD6_{15A}$ (bottom gel) primer in the genotype D_I primer mix are shown.

Figure 4:

Genotyping multiplex PCR of all strains from Vietnam with genotype H mix.

Figure 5:

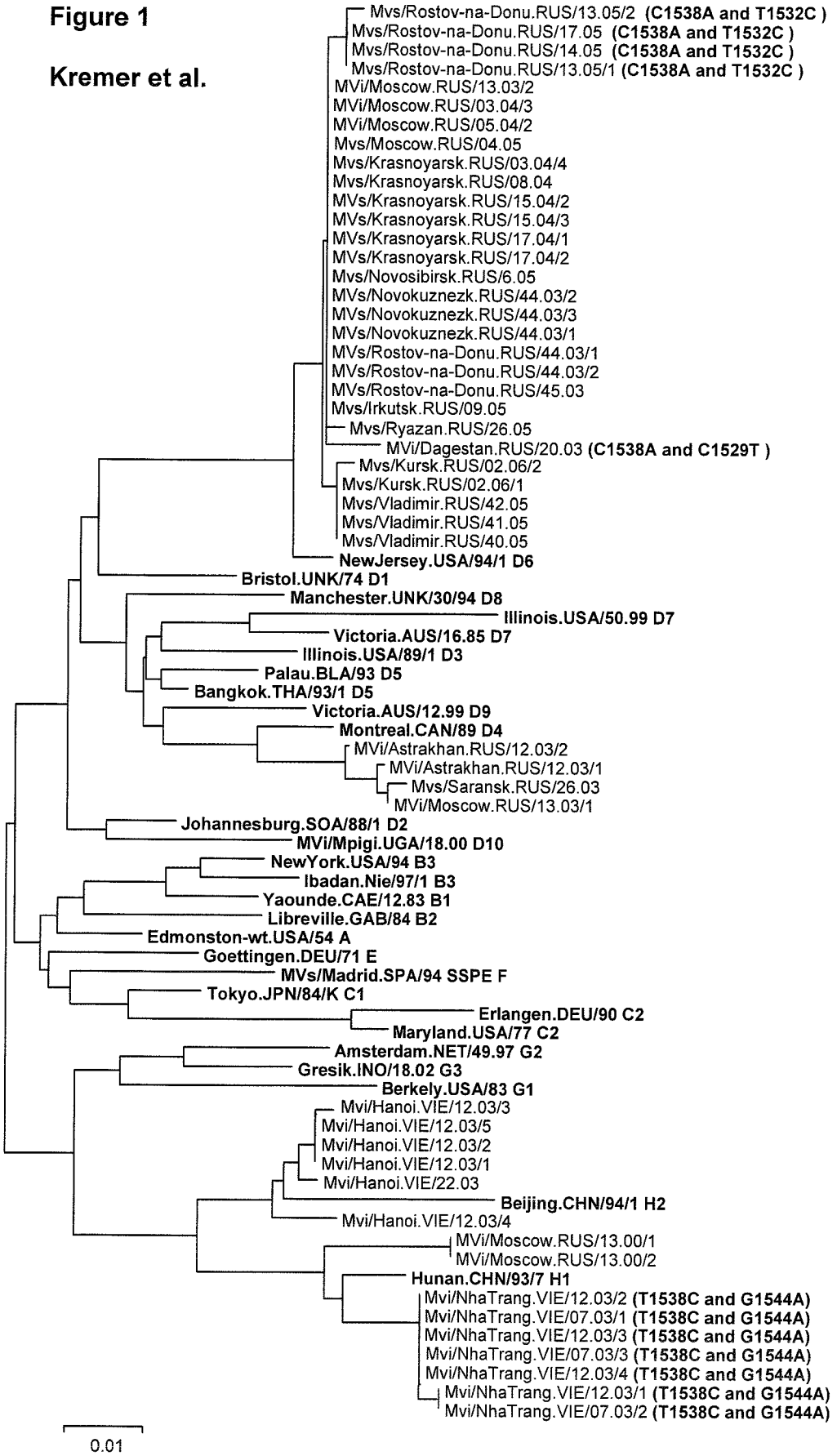
Multiplex PCR including genotype H1 (GeH1), genotype H2 (GeH2) and Nf1a sense primers as wells as Nr7a antisense primer. Results show all clade H strains from Vietnam as well as a clade A (Edmonston wild-type; U01987) and a clade B (MVi/Ibadan.NIE/97/1; AJ232203) strain.

Figure 6:

Genetic relationship of the 450 C-terminal nucleotides of the measles virus N-gene between strains from Vietnam and the most closely related strains retrieved in Genbank. Strains from Vietnam detected in 2003 are shown in bold, those from 1998 are in italic.

Figure 1

Kremer et al.



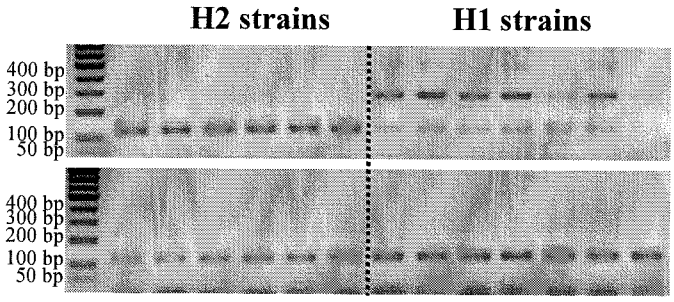


Figure 2

Kremer et al.

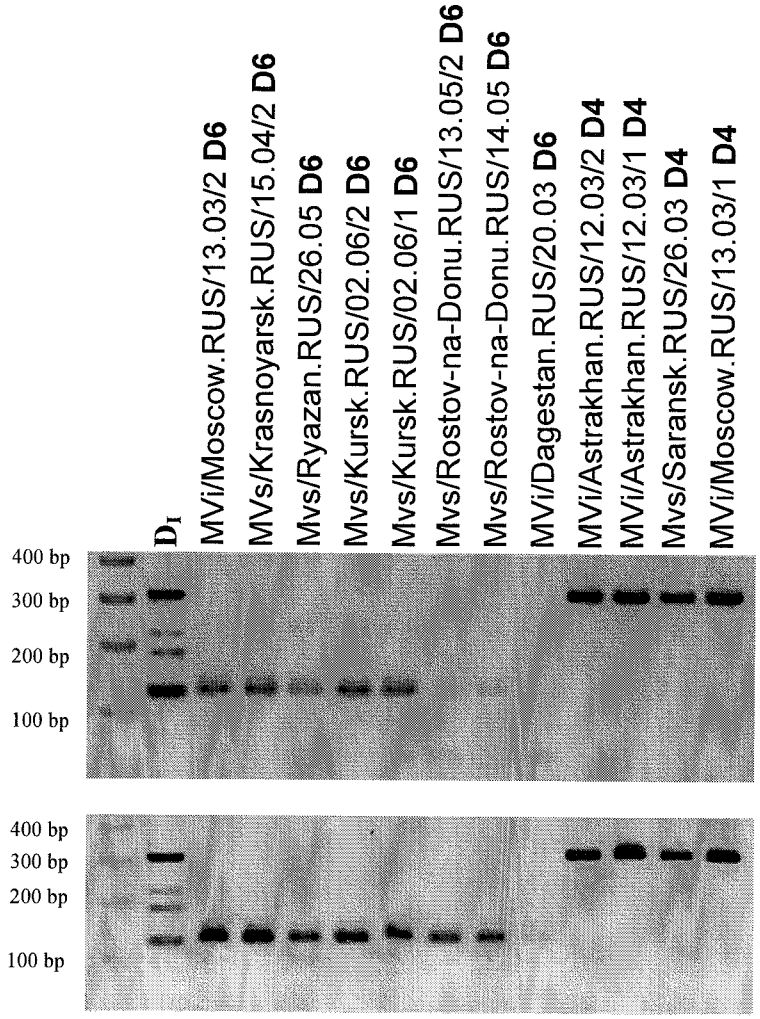


Figure 3

Kremer et al.

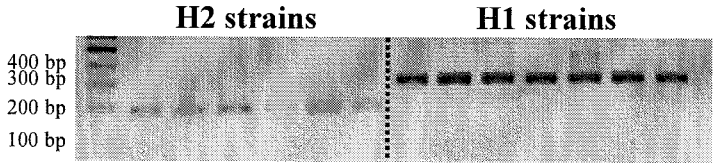


Figure 4

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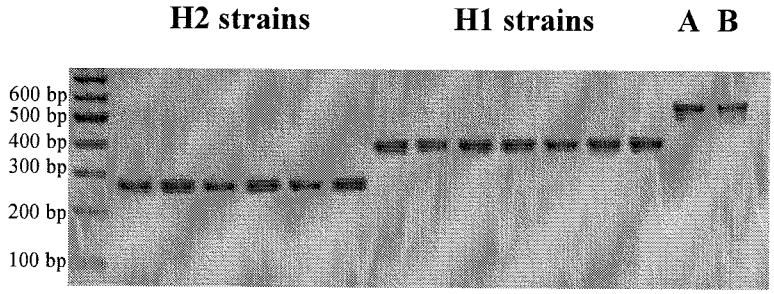

Figure 5

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Figure 6

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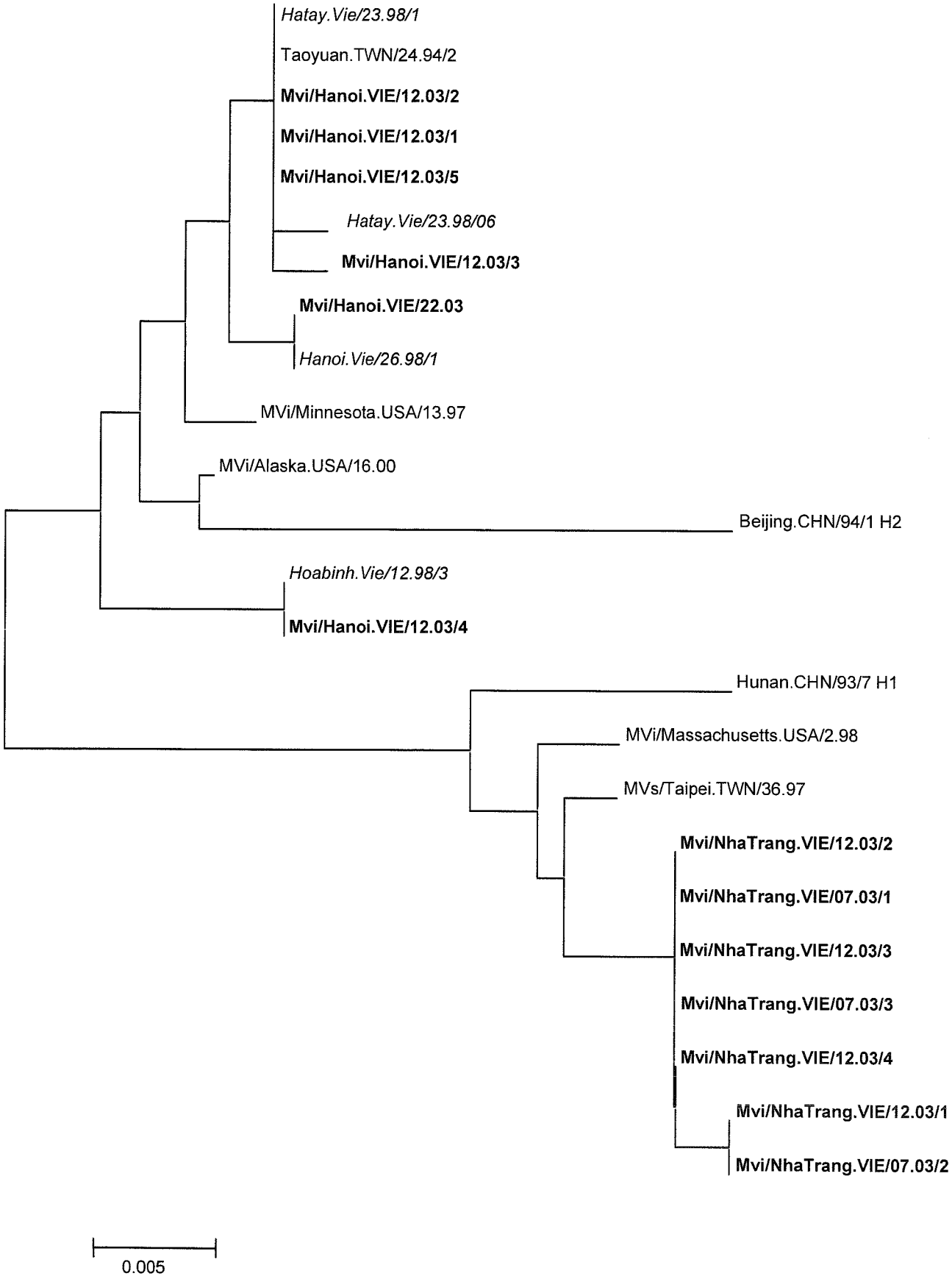

XII. Réduction globale de la rougeole et de la rubéole. Rapport sur la réunion du comité d'orientation de l'OMS pour la recherche concernant la rougeole et la rubéole, 2005

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Résumé de l'article

Lors d'une réunion à New Delhi, en Avril 2005, le comité d'orientation de l'OMS a évalué les progrès vers un contrôle global de la rougeole et de la rubéole. Sur la base d'informations échangées entre une centaine de participants provenant de différents continents et actifs dans le domaine de la santé publique et de la recherche biomédicale, le comité a mis au point des recommandations pour poursuivre la recherche sur la rougeole et la rubéole.
Entre 1999 et 2004 la couverture vaccinale contre la rougeole a augmenté de 71% à 76%. Dans de nombreux pays la circulation endémique du virus a pu être interrompue et maintenue à un niveau très bas. Le taux de mortalité global aurait diminué de 39% entre 1999 et 2003, grâce à des programmes de vaccination renforcés. La circulation du virus reste pourtant endémique dans de nombreux pays, en Afrique ainsi qu'en Asie du Sud-Est. Différents rapports sur l'incidence ainsi que les taux de mortalité due à la rougeole, présentés par les participants de pays ou la maladie est toujours endémique ont permis de développer des stratégies de contrôle améliorées. Dans ce contexte le comité d'orientation a motivé la recherche pour le développement de nouvelles stratégies de vaccination ainsi que l'amélioration des activités de surveillance par la mise en place de nouveaux outils diagnostics. L'étude de l'immunité contre la rougeole dans différentes populations et l'analyse rigoureuse d'épidémies devraient accompagner les stratégies de contrôle. Enfin, les avantages et désavantages d'une éradication globale du virus ont été discutés.
Puisque le contrôle de la rubéole et du syndrome de rubéole congénitale (SCR) sont moins avancés, les recommandations dans ce domaine ciblaient principalement le développement de stratégies de vaccination. Dans ce but, l'importance du syndrome de rubéole congénitale (SCR) a été comparée dans différentes régions du monde. Plusieurs analyses de coût-efficacité, ont permis de promouvoir l'introduction d'un programme de vaccination contre la rubéole dans plusieurs pays qui n'en avaient pas.
Aussi longtemps que les deux virus en question continuent de circuler, les stratégies de contrôle et de surveillance devront être optimisées. Ainsi la

méthode RT-PCR a été évaluée comme alternative pour le diagnostic du SCR. De même, plusieurs études ont étudié la détection d'anticorps IgM et IgG contre la rougeole et la rubéole dans du sang séché sur papier filtre ainsi que du fluide buccal. Le comité d'orientation a considéré que ces nouveaux outils devraient être d'avantage évalués avant leur utilisation en routine.

Reducing global disease burden of measles and rubella: Report of the WHO Steering Committee on research related to measles and rubella vaccines and vaccination, 2005

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Abstract

The WHO Steering Committee reviewed and evaluated the progress towards global control of measles and rubella and provided guidelines for future research activities concerning both diseases during its last meeting in New Delhi, in April 2005. Global measles vaccination coverage increased from 71% in 1999 to 76% in 2004 and indigenous transmission was interrupted or kept at very low levels in many countries. However, Africa and Southeast Asia continue to experience endemic transmission and high mortality rates, despite a global mortality reduction of 39% between 1999 and 2003. On the basis of reports from countries with continued indigenous measles virus transmission, future control strategies as well as advantages and potential drawbacks of global measles eradication were discussed. Similarly the burden of rubella and congenital rubella syndrome (CRS) as well as the cost-effectiveness of rubella vaccination was assessed using different methods in several countries without vaccination programs. As measles and rubella viruses continue to circulate surveillance and control strategies need further optimisation. RT-PCR was considered as an alternative method for laboratory diagnosis of CRS. The value of dried blood spots and oral fluid as alternative samples for measles and rubella IgG and IgM detection and genotype determination was evaluated. However further validation of these methods in different settings is required before their routine use can be recommended.

Running title: WHO Steering Committee Report for Measles and Rubella, 2005

Keywords: dried blood spots; oral fluid; congenital rubella syndrome

1. Introduction

On October 26th 1977, the last naturally infected case of smallpox was reported from Merka, Somalia. A world-wide coordinated effort under the leadership of the World Health Organization lead to the eradication of this dreadful disease. Despite recurrent set-backs poliomyelitis is also well on the way to elimination. The transmission of indigenous measles virus (MV) has been interrupted in the Americas, and in many other countries where outbreaks result solely from imported cases. Recently, tremendous progress was made in Africa and Asia to further reduce measles-related mortality. The burden of congenital rubella has been largely underestimated in developing countries [1], but WHO initiatives have renewed interest in this debilitating condition [2,3,4]. In order to improve laboratory surveillance for measles and rubella, WHO has set up a world-wide Laboratory Network for Measles and Rubella [5]. In April 2005, the WHO Steering Committee on Research related to Measles and Rubella Vaccines met in New Delhi for its annual meeting to review and evaluate progress towards global control of these two diseases.

2. Measles

2.1. Public health significance of measles

WHO [6] estimates that measles is responsible for 4 % of the 6 million annual deaths in children under-five. 98% of these deaths occur in developing countries. Thus, despite progress in global control, measles continues to be a serious condition and a leading cause of childhood death, particularly in developing countries. In 2004, WHO reported an estimated 76% coverage of

measles containing vaccines world-wide and 51% of countries reached ≥ 90% MCV coverage in all districts. Most countries have provided a second opportunity for measles vaccination, either by introducing a routine two-dose schedule or by mass campaigns. With 30 million estimated annual cases [7], most of them unvaccinated, MCV is still under-utilized.

2.2. Progress in reducing measles mortality

In 2001, WHO and UNICEF developed a 5-year strategic plan to reduce measles global mortality by 50% by the year 2005, compared to 1999 levels [8]. In regions with established measles elimination goals, the objective was to achieve and maintain interruption of indigenous measles transmission. The plan targeted 45 priority countries with the following major strategies: (i) high routine vaccination coverage (≥ 90%) in every district; (ii) providing a second opportunity for measles immunization, mostly through supplementary immunization campaigns; (iii) improving surveillance; (iv) improving case management including vitamin A supplementation and antibiotic treatment if needed.

Global vaccination coverage increased from 71% in 1999 to 76% in 2004, but Africa and Southeast Asia lag behind with an increase from 50 to 66 % coverage in the former and 59% to 63 % in the latter. In 2004, only nine of the 45 target countries offered no second opportunity for measles vaccination. In 2003 global measles mortality was estimated at 530.000 deaths, a 39% reduction from 1999 (Figure 1). Indirect indicators suggested that the largest reduction in mortality was achieved in the WHO African Region [9], largely by implementing the above 4 components of the WHO-UNICEF strategy. Otten et al. [10] reported a decline in annual measles deaths of about 20% (90,043 of

454,000) as a result of supplementary immunisation activities (SIA) in 19 African countries between 2000 and 2003.

2.3. Progress in eliminating indigenous measles transmission

Enormous progress towards measles elimination has been made in the Americas. In 1994, a goal was set to eliminate indigenous measles from the Western Hemisphere by 2000 [11]. Numbers of cases rapidly declined from 1990 to 1996. One year later a large outbreak started in São Paulo, Brazil and spread to Argentina and Bolivia. Major vaccination efforts led by these countries reduced numbers of cases reported in the region to 1754 and 537 cases by 2000 and 2001, respectively. Few countries had continuing virus transmission [12] and not a single child has died from measles during the last three years. Molecular tools demonstrated that endemic measles transmission has been interrupted, but cases imported from other regions and residents who become infected abroad continue to be a problem [13,14]. These achievements were only possible thanks to the strong commitment from health authorities to implement and sustain the above components of measles control.

In the European Region and other countries such as Australia, Mongolia, New Zealand, Philippines, the Pacific Island Nations and the Arab Gulf States, measles transmission has been interrupted or is at very low levels [15]. Also other regions have set elimination goals: the WHO European Region by 2007 and the Eastern Mediterranean region by 2010. The Western Pacific Region plans to eliminate measles by 2012 [16].

2.4. Countries with indigenous measles transmission

Large countries such as *Nigeria, India and Pakistan* continue to sustain large numbers of measles-related deaths (Dr BS Hersh). In 2003, for instance, India reported more than 47,000 measles cases, while the 115 measles-related deaths are likely to be an underestimate. The country has used monovalent measles vaccine at 9 months of age since 1986; MMR is used only in the private sector. Reported coverage has been consistently high (>80%), but the estimated coverage is much lower (40-70%), and varies between states.

Other areas such as *Niger* still report large outbreaks (Dr C Dubray). From November 2003 to June 2004, 11,073 cases were reported. 75% of cases and 86% of deaths occurred in children under five. Attack rates were highest among the 6-9 month olds. Overall the case fatality rate (CFR) was 1.8%. CFR among under-fives and 12-35 month olds was as high as 2.7% and 4.6% respectively. CFR was much higher (20%) among those admitted to hospital. The most prevalent complications were pneumonia (66%) and diarrhea (61%) followed by ear discharge (12.4%). This large outbreak was due to a failure to vaccinate, poor surveillance and an underestimation of CFR. In December following the outbreak, 94% of 9 months to 14 year old children were vaccinated during a nationwide SIA. In the long term, routine vaccination coverage must be improved and a second opportunity for vaccination provided.

In *Nepal*, Joshi et al. conducted a community-based, retrospective study of a national, representative sample of 37 measles outbreaks (>4 epidemiologically linked cases) that occurred between March and August 2004 and were reported by health institutions throughout Nepal. 5,303

measles cases with a median age of 6.4 years were reported; 25% and 60% of cases were 1-4 years and 5-15 years old, respectively. The crude CFR was 1.4%. The CFR was 8.9% for children <1 year, 2.9% for children 1-4 years, 0.5% for children 5-14 years, and 0.6% for children >14 years old. In response to these outbreaks, 9.5 million children were vaccinated.

Measles control is being integrated in many countries with other priority public health interventions, such as the distribution of insecticide-treated bed-nets, shown to reduce mortality due to malaria by one-third. Although there is a need to strengthen district-based immunization, current trends suggest that the goal of a 50% reduction of measles mortality by the end of 2005 compared to 1999 levels will have been met.

2.5. Estimating measles mortality

Given the weakness of disease surveillance and death registration in many parts of the world, routine reporting systems are not reliable enough to monitor measles mortality (Dr L Wolfson). A panel of experts met in January 2005 to discuss and advise WHO on methods for evaluating measles mortality reduction. It was recommended to use disease surveillance when data are reliable and mathematical models when data are limited. Despite their limitations, mathematical models based on strong assumptions can capture important parameters such as changes in vaccination coverage that influence mortality. Country-specific estimates of CFRs are a key driver in such models, however case-based surveillance with laboratory confirmation of suspected cases and documentation of cause-specific mortality should be implemented or improved in many countries (Anonymus 2005). In 19 African countries, Otten et

al. [10] reported an average decline in the number of reported measles cases of 91% resulting from supplementary immunisation activities (SIA) between 2000 and 2003, but whether or not the same reduction is seen in mortality is only assumed.

In this context the SC recommended the following research priorities: (i) further work on the estimation of measles CFR in different settings and standardization of the methodology; (ii) careful evaluation of the strengths and weaknesses of estimations of the proportion of child deaths due to measles (i.e., proportional mortality); (iii) identification of factors that affect CFR within and between countries; (iv) evaluation of obstacles to high and sustained vaccination coverage.

2.6. Towards measles eradication?

As progress in controlling and eliminating measles in many parts of the world is encouraging, the pros and cons of global measles eradication were discussed (A. Osterhaus). Favorable factors include (i) the absence of an animal reservoir, (ii) only rare persistent infections, (iii) the single virus serotype, (iv) the antigenic stability of the virus and (v) a safe and effective vaccine [17]. Challenges to measles eradication include (i) the impact of HIV endemicity on measles control, (ii) injection safety issues and the potential need for alternative routes of vaccination [18,19], (iii) political and economic obstacles, (iv) appropriate strategies for different country-specific situations, (v) the high infectivity of the measles virus requiring a population immunity of 90-95% [20], (vi) robust vaccine induced immunity. A. Osterhaus pointed out that vaccination may not necessarily be discontinued after eradication because of the risk of inadvertent reintroduction from a laboratory or from

persistently infected individuals, and the theoretical possibility of infection with another morbillivirus and bioterrorism. Furthermore, there are reports of non-specific benefits from receiving live-attenuated measles vaccine [21]. The benefits from eradication would include complete and sustained reduction of measles mortality and the absence of risk of virus transmission to pockets of seronegative individuals. The major risk of eradication may include a reduced vaccination compliance associated with the re-introduction of measles with high morbidity and mortality.

The SC recommended research in most of the above issues to obtain a better understanding of the implications of a measles eradication strategy. Thus recommendations included the investigation of immunological correlates of protection, the characterization of long-term memory immunity, the development of standardized assays for measuring T and B cell immunity; the need to investigate cases putatively infected by genotype A viruses and the pathogenic potential of viruses isolated from these cases; and continued research on alternative candidate measles vaccines, which are non-replicating, effective at an earlier age and can be combined with other vaccines.

3. Rubella

3.1. Introducing rubella vaccine into a national program

WHO has recommended that developing countries without rubella vaccination programs should assess the burden of rubella and congenital rubella, in order to consider whether vaccination should be introduced [3]. This can be done either by surveillance, seroprevalence, retrospective studies or

mathematical models. Guidelines for surveillance of rubella and CRS have been published [2]. Unlike for measles, there is no single strategy for rubella and CRS control. Two principal strategies may be considered: (1) selective vaccination of adolescent and adult females and (2) routine vaccination of all young children. The first strategy may prevent CRS, but does not control rubella. The second strategy can prevent CRS, control and eventually eliminate rubella and CRS. The use of these strategies is dependent on many issues such as infrastructure, goals of the program and funding [22].

In *South Africa*, (Dr L Blumberg) rubella vaccination is not part of the national program and in the private sector uptake is very limited. Most rubella cases occurred in children less than 10 years of age and 18% of cases are between 10-14 years old. Even though fewer than 10% of the cases are among persons aged >14 years, there is a concern about CRS in newborns; however, there is no CRS surveillance system to directly measure this problem.

China (Dr L Gao) is also investigating how to identify the best strategy for rubella vaccination, but data on rubella and CRS prevalence is limited. Mathematical models based on current demographic trends such as the one-child policy and declining birth rates, were used to compare different rubella vaccination strategies. Without vaccination CRS would increase 3-fold by 2050 as a result of the smaller birth cohorts. Childhood vaccination with < 50% coverage would also increase CRS. Rubella and CRS could be eliminated if vaccine coverage is ≥80%. Routine vaccination of 12-year old girls or mass campaigns in 15-40 year old women would reduce CRS, but would have no effect on rubella incidence. Mass vaccination of 2-14 year olds

would result in oscillating numbers of CRS cases with peaks above the no-vaccination level. These findings highlight the importance of changing demographic factors for the selection of the best rubella vaccination strategy.

Romania (Dr A Rafila) presented mathematical models based on epidemiological data of rubella of the last 45 years. As a result of decreasing birth rates, epidemic cycles increased from 5 years to 10 years with most cases among children <15 years old. Between 1994 and 1998, >70% of cases were >15 years old; before and after this period <20% of cases were in this age group. In 2003, rubella vaccine was selectively administered nationwide to adolescent girls in 8th grade, then, in 2004 rubella-containing vaccine was added to the routine childhood schedule in addition to the adolescent female vaccination. The different models showed that (i) 80% vaccination coverage of children with a single dose during the second year of life would control rubella and eliminate CRS eventually. (ii) A catch-up campaign among children 2-14 years old would reduce the time to elimination, but would not be necessary for elimination. (iii) Vaccination of adolescent girls and young women would reduce CRS by 3/4 without affecting rubella incidence. (iv) Including older women of childbearing age would have no additional benefit and would only add to the costs.

While the robustness of mathematical models to estimate CRS incidence may be questioned, the tools for direct monitoring of CRS are limited and costly (e.g. detection of rubella virus RNA in extracted cataract material; systematic hearing tests).

3.2. Cost-benefit assessments of rubella vaccination

In *Bhutan*, (T Dorji) laboratory diagnosis of rubella was introduced in 2003. Since then, rubella cases and several outbreaks have been confirmed. A retrospective study has documented a few clinical cases of CRS. Although rubella vaccination has been recommended, the best and most cost effective strategy and CRS surveillance are only being evaluated.

The *Republic of Maldives* (Dr N Ibrahim) does not vaccinate against rubella, but the disease became notifiable in 1999. Because of the relative isolation of the islands, the mean age of infection is 22 years, which is much higher than in most countries. Immunity among women of childbearing age is low, in particular outside of Male and the outer islands (<45%). The costs of the 2000 outbreak and the lifetime costs of an infant with CRS defects have been evaluated. It was estimated that a mass campaign with MMR vaccine in children aged 6-14 years as well as male and female adults would result in savings of US$4.3 million.

Hinman et al. [23] reviewed 22 cost analyses of rubella vaccination including 10 from developing countries. The cost-benefit of rubella and MMR vaccine was evaluated each in 5 studies and their cost-effectiveness in 2 studies from developed countries. According to these studies, rubella vaccination programs have not only resulted in significant reduction of morbidity and mortality, but also in substantial cost savings [23]. The benefit-cost (B:C) ratio for a routine childhood program in 3 developed countries was estimated to be 5.8 to 11.1. In the Americas, the annual cost to treat an infant with CRS ranged from about US$ 1000 in Guyana (<1997) and $2,291 in Panama (1989) to $13, 482 in Jamaica (1997). When compared with other

vaccine-preventable diseases such as *Haemophilus influenzae B* and Hepatitis B, rubella vaccination programs had a higher cost benefit ratio. These studies are important in assisting governments to decide in which programs to invest limited resources.

3.3. Monitoring rubella control activities

After introducing rubella vaccination into the national program, monitoring of its impact is critical because insufficient coverage tends to increase the age at which susceptibles become infected. Paradoxically the incidence of CRS may increase as a result of prevention. Countries that progress towards control of rubella, must be prepared to continuously adapt their vaccination strategy as exemplified below.

In *Italy* (Dr M. Ciofi degli Atti), a national rubella vaccination program was established in 1972 targeting pre-adolescent girls. In the early 1990s, this schedule was modified to include a childhood dose of MMR at 15 months. In 1999, the age of the first dose was lowered to 12-15 months and a second dose of MMR vaccine recommended at either 5 – 6 years or 11-12 years of age. In 2003, the vaccination coverage among children aged 24 months was 77%. The epidemiology of rubella has changed since the introduction of rubella vaccines. Outbreaks occurred every 4-5 years, the last one in 1997. The mean age of infection increased from 9.5 years between 1976-1980 to 12.3 years between 1998-2003. Nevertheless the incidence of rubella in women of childbearing age has decreased from 14.1 to 2.8 cases per 100,000 population, but there is no surveillance of CRS. In 2003, a national plan for elimination of measles and CRS by 2007 was established. Strategies include

the target of 95% vaccination coverage by 2 years of age; lowering the 2nd dose of MMR to 5-6 years of age; conducting a catch-up campaign for unvaccinated children and adolescents and vaccinating susceptible women of childbearing age. Surveillance should be improved by mandatory notification of rubella during pregnancy and of congenital rubella.

In *Sri Lanka* (Dr P. Palihawadana) the introduction of rubella vaccine was prompted by an increase in rubella cases and documented CRS cases. In 1996, rubella vaccine was first administered to adolescent girls and women of childbearing age. In 2001, two additional age cohorts (3 and 8 years of age) were added to the vaccination strategy. In 2001 through 2004, 72-82% of women of childbearing age were vaccinated. This correlates with a seroprevalence study conducted in 1999 in the Kalutara District (Sri Lanka; 24). In children, coverage increased in 2003/2004 from 89 to 98%. Future plans include strengthening of laboratory confirmation of rubella and CRS as well as improving vaccination coverage by school-based programs.

Both countries have monitored the impact of their vaccination program by assessing their coverage data and have adapted their control strategies, which is particularly important for CRS control.

3.4. Surveillance of rubella and CRS

Studies from Vellore, South *India* (Dr D. Brown) compared different methods for assessing the burden of CRS. Antenatal surveys in 1991 and 1998 showed that 8% of women were susceptible to rubella when 4 IU/ml was used as cut-off in a commercial enzyme immunoassay. Susceptibility was higher in the rural population than in urban women. Two age-stratified

community surveys in rural and urban areas showed that the highest force of infection was in 5-9 year-old children. The estimated incidence of CRS ranged from 32–97 per 100,000 live births, but active hospital-based surveillance of CRS between 1996-2001 identified only 22 cases (0-9.4 cases per 100,000 live births), only 6% of predicted cases. Eleven (50%) of these cases had cataracts. The community survey was the most informative, but was also the most complex and costly. Rash/fever surveillance in pregnant women was recommended to identify children at risk of CRS. Another study in three eye-hospitals in South India showed that 11.7 – 20.8% of female hospital staff were susceptible to rubella [25].

Rubella IgM testing is the most convenient method for the confirmation of CRS, but commercially available tests have not been evaluated for this purpose [26] and some lack sensitivity [27]. This was also confirmed in studies in India. In addition, rubella IgM test kits are expensive and are not always available in developing countries. The SC recommended that available rubella IgM tests should be evaluated for diagnosis of CRS, using sera from children of different ages.

In *Morocco* (Dr R Ahmed), the CRS burden was assessed in a retrospective study [28]. Sixty-two cases of CRS by WHO case definition were identified from medical records, 148 from disability records and 15 among deaf schoolchildren. No laboratory confirmation was carried out. The yearly incidence of CRS was estimated to be 8.1-12.7 cases per 100,000 live births, a figure consistent with past estimates from the US and UK. The authors suggested that ophthalmology and cardiology departments may be best placed for finding cases of CRS. Retinal surveys in deaf schools were also

convenient. The study concluded that rubella vaccine should be used in Morocco, where 15-34% of women at childbearing age are susceptible to rubella [reviewed by Bloom, 28].

Another recent report estimated the CRS burden in Iran in 1995/1996 by evaluating the proportion of children with sensorineural hearing loss attributable to rubella [29]. A case-control study tested 113 children in schools for the deaf and 112 controls aged 1 to 4 years for rubella antibodies. 19.5% cases and 8.9% controls had rubella antibodies. On the basis of these findings the proportion of cases with deafness attributable to rubella was estimated to be 12%, and CRS prevalence in Iran was calculated as 20 per 100,000 children. Iran has recently introduced childhood MMR vaccination and in a mass campaign about 32 million people aged 5-25 years were vaccinated with MR vaccine. A surveillance program should now be established to monitor the impact of vaccination, as recommended by WHO [2].

These reports show that identification of CRS cases is difficult and labor intensive. As the definition of CRS includes several defects, the specificity may be high but the sensitivity low.

3.5. Diagnostic value of RT-PCR for prenatal diagnosis of congenital rubella

The diagnosis of primary rubella infection is based on detection of specific IgM and/or rubella IgG seroconversion and rubella IgG avidity testing. The interpretation of results may be complicated by false-positivity, cross-reacting IgM or persistent rubella IgM [26,30]. When serological results are inconclusive, or primary infection occurs in the early second trimester or

reinfection is confirmed in the first trimester, a prenatal diagnosis may be required to determine the risk of CRS for the fetus. RT-PCR can be used to detect RV RNA in amniotic fluid, chorionic villus biopsies and fetal blood, but few studies have been published [26]. Dr L. Grangeot-Keros (Paris, France) reported results obtained from 110 amniotic fluids and 29 fetal blood samples from pregnant women with confirmed primary rubella infections. In this study, RT-PCR was a valuable tool for prenatal diagnosis, with a sensitivity of 83-95% and a specificity of 100% [31]. Detection of rubella IgM in fetal blood obtained by cordocentesis is also a valuable test for prenatal diagnosis, but the collection of fetal blood is technically more demanding [26; L Grangeot-Keros, personal communication 2005]. It is important that specimens are transported at 4°C and are collected no earlier than seven weeks after infection [reviewed by Best and Enders, 26].

4. Genotyping of measles and rubella

Laboratory surveillance is a key component of measles and rubella control programs. It includes serological confirmation of suspected cases and genetic characterization of endemic/epidemic viruses. Genotyping is useful for molecular epidemiology and surveillance and allows tracking of virus transmission, e.g. to demonstrate elimination of indigenous MV and RV from the USA [32,33]. Genetic characterization of MV has been proven to be a valuable tool for measuring the effectiveness of measles control programs [34]. In a given geographic region, a genotype is considered endemic if it is consistently found in the same region over an extended time period. If different genotypes are associated with limited outbreaks and/or sporadic

cases, these are likely to result from multiple importations of viruses into the region rather than from circulating endemic viruses [35,36]. Both for MV [37] and RV [38], WHO recommends a standardized nomenclature.

Measles virus. Genotypes are determined by phylogenetic analysis of the 450 C-terminal nucleotides of the nucleoprotein gene and/or of the entire hemagglutinin gene [39]. There are currently 23 recognized genotypes (A, B1-3, C1-2, D1-10, E, F, G1-3, H1-2; 39), most of which are actively circulating in more or less confined geographic regions with possible long distance importations [36,41]. The SC recommended to further improve molecular tools for detection and characterization of MV and to continue the study of virus transmission patterns via molecular epidemiology, to help monitor measles control programs.

Rubella virus. Various regions of the E1 gene have been proposed by different authors for genetic characterization. A window of 739 nucleotides (nt 8731-9469) is recommended for routine molecular analysis [38]. Two major phylogenetic groups of RV designated clades 1 and 2 have been described, differing by 8-10% of their nucleotides. Seven genotypes designated with upper-case letters (1B, 1C, 1D, 1E, 1F, 2A and 2C have been accepted. Reference viruses are available for these genotypes. Three provisional genotypes (1a, 1g and 2c) have also been described. Clade 1 viruses currently exist worldwide, while clade 2 viruses have been found predominantly in Asia, with occasional isolates from Europe [38]. Clade 2 viruses have not been detected in the Western Hemisphere (Figure 2).

Dr W. Xu (*China*) reported on the epidemiology and circulating genotypes of rubella in China. Locally-produced BRD-2 rubella vaccine was

approved by the Chinese regulatory authorities in 1998 and is now used routinely in many provinces. Sentinel surveillance sites reported a major peak in rubella incidence in 1993 (98 cases/100,000) and a few outbreaks in Shandong province in 2001. Sequencing of 601 bp of the E1 coding region (nt 8869-9469) of 42 isolates collected between 1999 to 2003 from Anhui, Henan and Shandong provinces, identified genotypes 1E, 1F, 2A and 2B. In 2001, rubella cases caused by both genotypes 1F and 2A occurred within 100 km from each other in Shandong province. The heterogeneity of RV is much greater than that of measles viruses isolated between 1993 and 2005 in China suggesting that measles control is a ahead of rubella control.

The SC recommended that genetic analysis and isolation of RV should be continued, as the circulating viruses from many countries have not been characterized. Specimens for virus isolation and /or sequencing should be obtained at the same time as serum for serological diagnosis and sent to the local WHO network laboratory [5].

5. Alternative sampling for diagnosis of measles and rubella/CRS

Case confirmation relies on IgM testing using validated ELISA tests (e.g. Dade Behring) for measles. Serum is considered the preferred sample for case confirmation but less invasive sampling techniques are being evaluated [5]. Dried blood spots (DBS) and oral fluid (OF) have both been confirmed to be suitable for IgM testing [42,43,[44]]. DBS are collected by finger prick onto filter paper (Whatman 903, Schleicher&Schuell), which is easier and considered less invasive than venupuncture [45]. As DBS are not considered "Dangerous Goods" for transport purposes, packaging and transportation to

the laboratory is also simplified. DBS are reconstituted in the laboratory and tested for IgM by ELISA [42]. OF collection is acceptable to patients, particularly children, as it is non-invasive and painless. OF consists of gingival crevicular fluid; therefore more sensitive assays are required [46]. Best results were obtained when OF was collected using a special device [47] and tested for IgM by capture EIA (e.g. Microimmune, Brentford, UK).

The SC recommended further optimisation of elution techniques and further development of RT-PCR for use with DBS.

Measles. DBS tested with a modified Behring ELISA has a sensitivity of 97-100% and a specificity of 91-100% for measles IgM, OF using Oracol and the Microimmune IgM assay has a sensitivity of 96-99% and a specificity of 84-100%.

Typically, genetic typing relies on PCR of viral RNA from virus cultures or directly from peripheral blood lymphocytes, urine and nasopharyngeal swaps. For virus isolation Vero-SLAM cells have been recommended to replace B95a cells, which actively produce Epstein-Barr-Virus [40]. OF can also be used for virus isolation and RNA detection by PCR [48]. In DBS the lower sensitivity for RNA detection can be partially offset by a larger amount of DBS and a nested PCR [49].

OF was also used to evaluate seroconversion after vaccination and to detect and genotype measles virus in Ethiopia [50]. In this setting, OF provided a convenient, non-invasive sample for both IgG-serology and virus surveillance. As there was some concern about the sensitivity of IgG in vaccinees with lower IgG levels than late convalescent donors, further

evaluation of the methodology in different settings is needed.

The Subcommittee concluded that regions without measles control, with only minimal laboratory support and without adequate access to expertise/supplies for conventional specimen collection, may benefit from the use of DBS or OF. Whenever possible, conventional sampling methods should be performed on a few cases of an outbreak. Regions with controlled measles but periodic outbreaks could initiate DBS or OF sampling in parallel with conventional methods. Regions in elimination phase with few outbreaks would not generally benefit from alternative sampling methods and should be evaluated on a case-by-case basis. Accordingly, the SC has asked WHO to continue field trials of these techniques in different stages of measles control.

Rubella. A commercial test for rubella IgM in OF is under development, and field trials are required. Dried blood spots can be used to detect rubella antibodies [51,52]. OF can be used to detect rubella IgM in both postnatally acquired rubella and CRS, but is not suitable for rubella IgG detection in adults [53,54,55]. RNA can be extracted and used for detection of RV by RT-PCR [56]. OF has been found to be better for nucleic acid extraction than serum or blood due to lack of PCR inhibitors.

The SC considers the preliminary studies as promising and recommends further evaluation of OF. WHO should produce protocols for use of these alternative sampling methods, including their limitations. Further research should also determine whether this RV sequences can be reliably obtained from alternative specimens, such as serum, DBS or OF.

6. Concluding remarks

Following the guidelines of the Strategic plan for measles control 2001-2005 developed by WHO and UNICEF, worldwide measles morbidity and mortality have been significantly reduced. The Steering Committee has developed research guidelines to support future measles reduction strategies. These include the evaluation of population immunity, outbreak investigation, design of vaccination strategies as well as the optimization of surveillance activities including the implementation and validation of new diagnostic tools. The surveillance and control of rubella and CRS lags behind measles control. Therefore, the guidelines concerning rubella and CRS focused on optimizing the tools to assess the burden of disease, to identify the best vaccination strategy for a given setting, and to improve diagnostic methods, particularly for the diagnostics of CRS.

References

[1] Cutts FT, Robertson SE, Diaz-Ortega JL, Samuel R. Control of rubella and congenital rubella syndrome (CRS) in developing countries, Part 1: burden of disease from CRS. Bull WHO 1997;75 55-68.

[2] World Health Organization. Guidelines for surveillance of congenital rubella syndrome and rubella. Field test version. WHO/V&B/99.22. World Health Organization, Geneva, 1999.

[3] World Health Organization. Preventing congenital rubella syndrome. Weekly Epidemiological Record. 2000;75:290-95.

[4] Best JM, Castillo-Solorzano C, Spika JS, Icenogle J, Glasser JW, Gay NJ, et al. Reducing the global burden of congenital rubella syndrome: report of the World Health Organization Steering Committee On Research Related To Measles and Rubella Vaccines and Vaccination, 2004. J Infect Dis 2005;192:1890-97.

[5] World Health Organization. Global measles and rubella laboratory network - update. Weekly Epidemiological Record 2005a;80:384-88.

[6] World Health Organization. The world health report 2005: Make every mother and child count. Geneva: WHO

[7] WHO/UNICEF. Joint statement on strategies to reduce measles mortality worldwide. World Health Organization, Geneva, 2001.

[8] WHO/UNICEF. Measles: mortality reduction and regional elimination. Strategic plan 2001-2005. World Health Organization, Geneva, 2001.

http:/www.who.int/vaccines-documents (accessed September 2005).

[9] World Health Organization. www.who.int/immunization monitoring/diseases /measles/en/index.html (accessed November 2005).

[10] Otten M, Kezaala R, Fall A, Masresha B, Martin R, Cairns L et al.. Public-health impact of accelerated measles control in the WHO African Region 2000-2003. Lancet 2005;366(9488):787-88.

[11] Quadros CA, Hersh BS, Nogueira AC, Carrasco PA, Silveira CM. Measles eradication: experience in the Américas. Bull WHO 1998;76(Suppl 2):47-52.

[12] Quadros CA, Izurieta H, Carrasco P, Brana M, Tambini G. Progress toward measles eradication in the Region of the Américas. J Infect Dis 2003;187(Suppl 1):102-09.

[13] Katz SL, Hinman AR. Summary and conclusions: measles elimination meeting, 16-17 March 2000. J Infect Dis 2004;189(Suppl 1):43-7.

[14] Preventable measles among US residents, 2001-2004. Centers for disease control and prevention (CDC). Morbidity and Mortality Weekly Report 2005;54(33):817-20.

[15] Anoynmous 1999. World Health Organization. Measles. Progress towards global control and regional elimination, 1998-1999. Weekly Epidemiological Record 1999;74(50):429-34.

[16] CDC. Global Measles and Rubella Laboratory network, January 2004-

June 2005. MMWR 2005;54(43):1100-04.

[17] Olive JM, Aylward BR, Melgaard B. Disease eradication as a public health strategy: is measles next. World Health Statistics Quarterly 1997;50:185-87.

[18] Cutts FT, Henao-Restrepo AM, Olive JM. Measles elimination: progress and challenges. Vaccine 1999;17:47-52.

[19] Strebel P, Cochi S, Grabowsky M, Bilous J, Hersh BS, Okwo-Bele JM. The unfinished measles immunization agenda. J Infect Dis 2003;187(Suppl 1): 1-7.

[20] Gay NJ. The theory of measles elimination: implications for the design of elimination strategies. J Infect Dis 2004;189(Suppl 1):27-9.

[21] Aaby P, Samb B, Simondon F, Seck AM, Knudsen K, Whittle H. Non-specific beneficial effect of measles immunization: analysis of mortality studies from developing countries. BMJ 1995;311:481-85.

[22] Robertson SE, Cutts FT, Samuel R, Diaz-Ortega JL. Control of rubella and congenital rubella syndrome (CRS) in developing countries, part 2: vaccination against rubella. Bull WHO 1997;75:69-80.

[23] Hinman AR, Irons B, Lewis M, et al. Economic Analyses of rubella and rubella vaccines: a global review. Bull WHO 2002;80:264-70.

[24] Palihawadana P, Wickreemasinghe AR, Perea J. Seroprevalence of rubella antibodies among pregnant females in Sri Lanka. Southeast Asian J Trop Med 2003;34:398-404.

[25] Vijayalakshmi P, Anuradha R, Prakash K, Narendran K, Ravindran M, Prajna L et al. Rubella serosurveys at three Aravind Eye Hospitals in Tamil Nadu, India. Bull WHO 2004;82:259-64.

[26] Best JM, Enders G. Laboratory Diagnosis of Rubella and Congenital Rubella. In: Banatvala JE, editor. Rubella Monograph. London, Elsevier 2006. In press.

[27] Corcoran C, Hardie DR. Serologic diagnosis of congenital rubella: a cautionary tale. Ped Infect Dis J 2005;24(3):286-87.

[28] Bloom S, Ahmed R, Berraho A, Zniber L, Bouazzaoui N, Zaghloul K et al. Congenital rubella syndrome burden in Morocco: a rapid retrospective assessment. Lancet 2005;365:135-41.

[29] Sadighi J, Eftekhar H, Mohammad K. Congenital rubella syndrome in Iran. BMC Infect Dis 2005;5:44.

[30] Best JM, O'Shea S, Tipples G, Lee SHS, Davies N, Al-Khusaiby S, et al. Interpretation of rubella serology in pregnancy -- pitfalls and problems. Brit Med J 2002;325:147-48.

[31] Macé M, Cointe D, Six C, Levy-Bruhl D, Parent du Chatelet I, Ingrand D, et al. Diagnostic value of reverse transcription-PCR of amniotic fluid for prenatal diagnosis of congenital rubella infection in pregnant women with confirmed primary rubella infection. J Clin Microbiol 2004;42(10): 4818-820.

[32] Progress toward measles elimination--region of the Americas, 2002-

2003. Morbidity and Mortality Weekly Report 2004;53(14):304-06.

[33] Reef SE, Frey TK, Theall K, Abernathy E, Burnett CL, Icenogle J et al. The changing epidemiology of rubella in the 1990s. JAMA 2002;287:464-72.

[34] Mulders MN, Nebie YK, Fack F, Kapitanyuk T, Sanou O, Valea DC, et al. Limited diversity of measles field isolates after a national immunization day in Burkina Faso: progress from endemic to epidemic transmission? J Infect Dis 2003;187(Suppl 1):277-82.

[35] Hanses F, van Binnendijk R, Ammerlaan W, Truong AT, de Rond L, Schneider F, et al. Genetic variability of measles viruses circulating in the Benelux. Arch Virol 2000;145(3):541-51.

[36] Rota PA, Rota JS, Redd SB, Papania MJ, Bellini WJ. Genetic analysis of measles viruses isolated in the United States between 1989 and 2001: absence of an endemic genotype since 1994. J Infect Dis 2004;189(Suppl 1):160-64.

[37] World Health Organization. Expanded Programme on Immunization (EPI). Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. Weekly Epidemiological Record 1998;73(35):265-69.

[38] World Health Organization. Standardization of the nomenclature for genetic characteristics of wild-type rubella viruses. Weekly Epidemiological Record 2005b;80:126-32.

[39] World Health Organization. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference strains. Weekly Epidemiological Record 2003;78(27):229-32.

[40] World Health Organization. New genotype of measles virus and update on global distribution of measles genotypes. Weekly Epidemiological Record 2005;80(40):347-51.

[41] Muller CP, Mulders NM, Molecular epidemiology of measles virus. The Molecular Epidemiology of Human Viruses, e.K. Thomas Leitner, Kluwer academic Publishers, Boston. 2002;237-72.

[42] Riddell MA, Leydon JA, Catton MG, Kelly HA. Detection of measles virus-specific immunoglobulin M in dried venous blood samples by using a commercial enzyme immunoassay. J Clin Microbiol 2002;40(1):5-9.

[43] El Mubarak HS, Yuksel S, Mustafa OM, Ibrahim SA, Osterhaus AD, De Swart RL. Surveillance of measles in the Sudan using filter paper blood samples. J Med Virol 2004;73(4):624-30.

[44] Ibrahim SA, Abdallah A, Saleh EA, Osterhaus AD, De Swart RL. Measles virus-specific antibody levels in Sudanese infants: a prospective study using filter-paper blood samples. Epidemiol Infect 2006;134(1):79-85.

[45] Chakravarti A, Rawat D, Yadav S. Whole blood samples as an alternative to serum for detection of immunity to measles virus by

ELISA. Diagn Microbiol Infect Dis 2003;47(4)563-7.

[46] Mortimer PP, Parry JV. Non-invasive virological diagnosis: are saliva and urine specimens adequate substitutes for blood? Rev Med Virol 1991;1:73-8.

[47] Vyse AJ, Cohen BJ, Ramsay ME. A comparison of oral fluid collection devices for use in the surveillance of virus diseases in children. Public Health 2001;115:201-07.

[48] van Binnendijk RS, van den Hof S, van den Kerkhof H, Kohl RH, Woonink F, Berbers GA, et al. Evaluation of serological and virological tests in the diagnosis of clinical and subclinical measles virus infections during an outbreak of measles in The Netherlands. J Infect Dis 2003;188(6):898-903.

[49] Katz RS, Premenko-Lanier M, McChesney MB, Rota PA, Bellini WJ. Detection of measles virus RNA in whole blood stored on filter paper. J Med Virol 2002;67(4):596-602.

[50] Nokes DJ, Enquselassie F, Nigatu W, Vyse AJ, Cohen BJ, Brown DW, et al. Has oral fluid the potential to replace serum for the evaluation of population immunity levels? A study of measles, rubella and hepatitis B in rural Ethiopia. Bull WHO 2001;79(7):588-95.

[51] Helfand RF, Keyserling HL, Williams I, Murry A, Mei J, Moscatiello C, Icenogle J, Bellini WJ. Comparative detection of measles and rubella IgM and IgG derived from filter paper blood and serum samples. J Med Virol 2001;65(4):751-57.

[52] Karapanagiotidis T, Riddell M, Kelly H. Detection of rubella immunoglobulin M from dried venous blood spots using a commercial enzyme immunoassay. Diagn Microbiol Infect Dis 2005;53:107-11.

[53] Perry KR, Brown WG, Parry JV, Panday S, Pipkin C, Richards A. Detection of measles, mumps and rubella antibodies in saliva using antibody capture radioimmunoassay. J Med Virol 1993;40:235-40.

[54] Eckstein MB, Brown DWG, Foster A, Richards AF, Gilbert CE, Vijayalakshmi P. Congenital rubella in south India: diagnosis using saliva from infants with cataract. Brit Med J 1996;312(7024):161.

[55] Nokes DJ, Nigatu W, Abebe A, Messele T, Dejene A, Enquselassie F, et al. A comparison of oral fluid and serum for the detection of rubella-specific antibodies in a community study in Addis Ababa, Ethiopia. Trop Med Internat Health 1998;3:258-67.

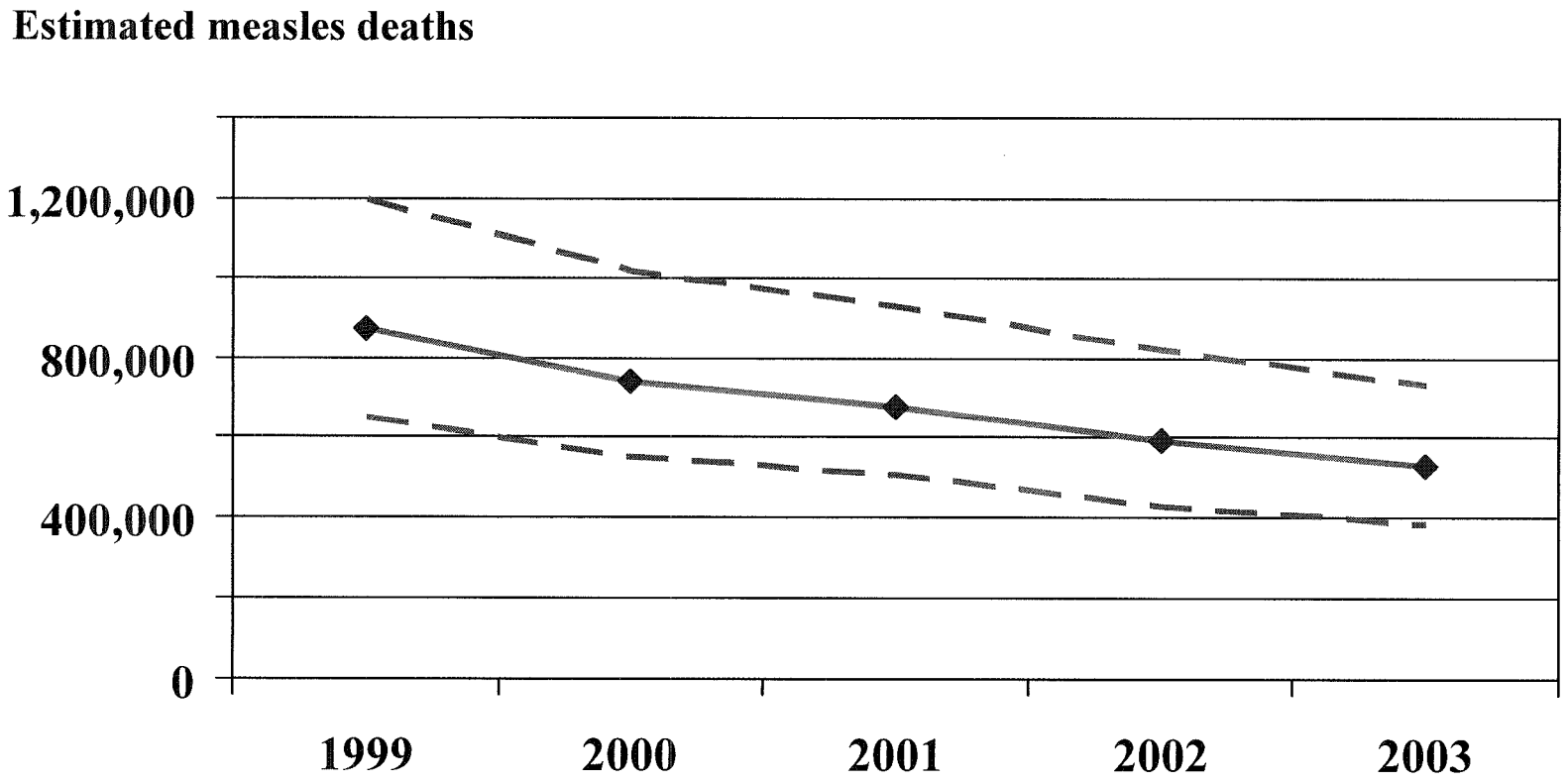
[56] Jin L, Vyse A, Brown DW. The role of RT-PCR assay of oral fluid for diagnosis and surveillance of measles, mumps and rubella. Bull WHO 2002;80:76-7.

Legend to Figures

Fig. 1. Trends in estimated annual global measles deaths with uncertainty levels 1999-2003. Provisional data for 2003. Source: World Health Organization.

Fig. 2. Geographic distribution of genotypes of rubella virus (1995-2005). Clade 1 viruses currently exist world-wide. Clade 2 viruses have not been found circulating in the Western Hemisphere.

Fig. 1.


Estimated measles deaths
1,200,000
800,000
400,000
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1999
2000
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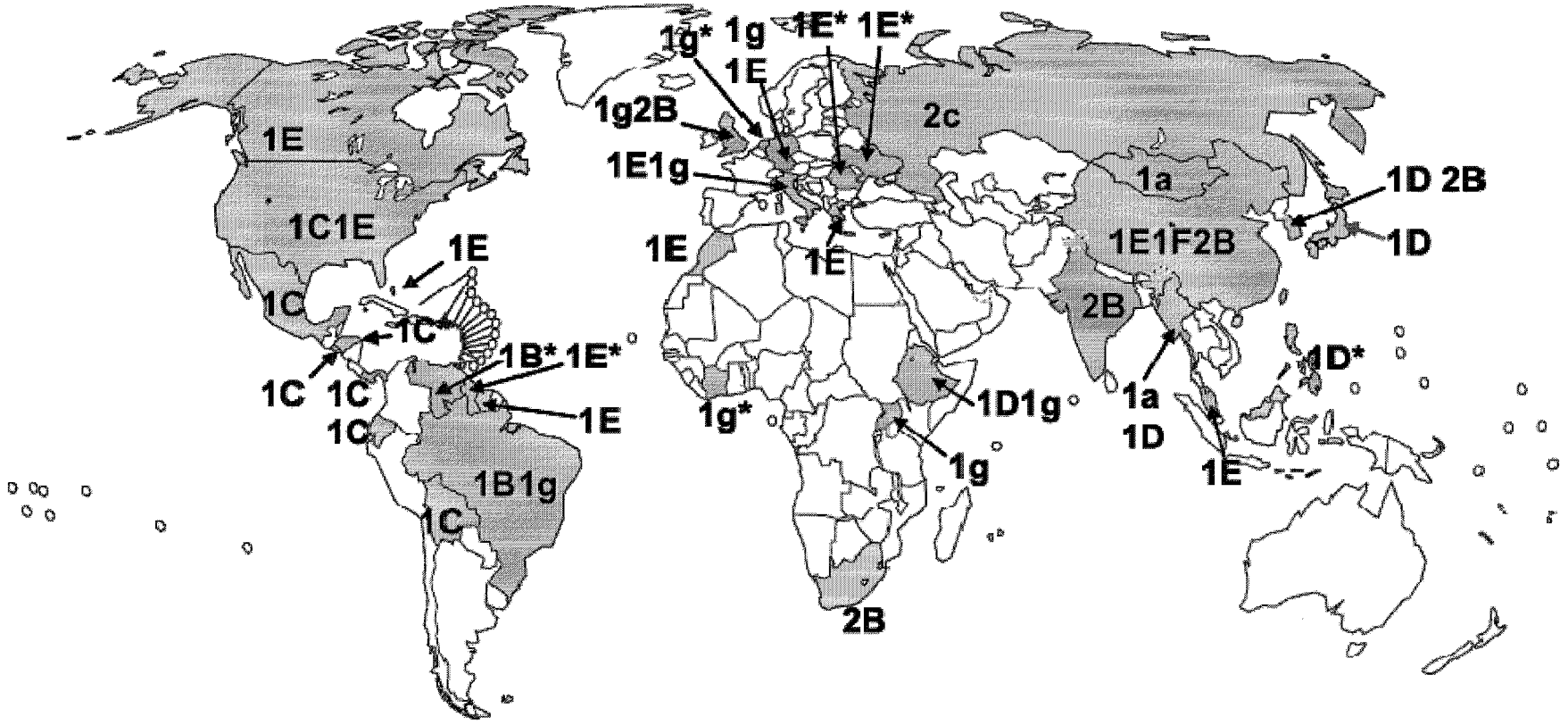
Fig. 2


1g*
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CONCLUSION

Afin d'améliorer le contrôle de la rougeole, l'OMS a établi différents plans stratégiques d'élimination régionale du virus. Par conséquent la circulation du virus de la rougeole a pu être interrompue dans plusieurs régions du monde, grâce à une application stricte des programmes de vaccination. Il est cependant important dans ces régions de maintenir une couverture de vaccination suffisante pour éviter la réintroduction du virus à partir d'une région où la circulation est restée endémique.

Plusieurs études ont montré que les taux d'anticorps antirougeole diminuent après vaccination. Nous avons étudié l'évolution des anticorps chez des adolescents vaccinés afin d'analyser si ce phénomène pourrait compliquer le contrôle de la maladie à long terme. Nous avons observé une perte d'anticorps accélérée lorsque les taux de départ étaient relativement élevés, alors que les taux relativement bas restaient plus stables. Par conséquent peu d'individus vaccinés risquent de devenir complètement séronégatifs au cours de leur vie, bien que les taux d'anticorps peuvent rapidement atteindre un niveau relativement bas. L'analyse des taux d'anticorps chez des convalescents réexposés à une souche sauvage de la rougeole a montré que la réexposition peut induire une stabilisation prolongée des taux d'anticorps. Quelques individus, plus faiblement protégés, ont même développé une réponse immunitaire secondaire, résultant dans une augmentation prolongée des taux d'anticorps. Ces événements indiquent que le virus de la rougeole peut infecter des individus séropositifs, lorsque les taux d'anticorps sont relativement bas. Les points précédents illustrent l'importance d'analyser avec rigueur les épidémies de rougeole des populations vaccinées dans le futur. Aujourd'hui une grande partie des populations est toujours protégée par infection naturelle. Cependant la proportion d'individus vaccinés augmente continuellement, et les stimulations immunitaires par des souches sauvages sont rares. Dans ces populations le niveau d'immunité devra être régulièrement analysé afin d'identifier des individus séronégatifs ou faiblement protégés. La détection d'anticorps dans le fluide buccal pourrait faciliter des études cross-sectionales pour identifier des groupes d'âges particulièrement susceptibles à une infection. Notre évaluation d'un test ELISA développé pour détecter les IgG spécifiques de la rougeole dans du

fluide buccal a cependant montré que la sensibilité de détection est significativement réduite par rapport au sérum.

A part la surveillance de l'immunité chez l'hôte, il est aussi important d'analyser les voies de transmission du virus pour améliorer le contrôle de la maladie. C'est pourquoi l'OMS a élaboré un protocole ainsi qu'une nomenclature standardisée pour attribuer chaque souche à un génotype précis. L'identification de six nouveaux génotypes lors des premières études d'épidémiologie moléculaire menées sur le continent asiatique a montré l'importance de caractériser régulièrement les souches circulant dans les différentes régions, afin de connaître la distribution globale des génotypes. D'après le protocole de l'OMS le génotype d'une souche inconnue est déterminé par séquençage d'une partie du génome. Il en découle que seuls les laboratoires équipés avec des facilités de séquençage, pouvaient identifier le génotype des virus. C'est la raison pour laquelle nous avons développé une méthode de génotypage plus simple et plus rapide, basée sur la technique PCR multiplex, pour faire la distinction entre les clades et génotypes du virus de la rougeole considérés actifs (A, B3.1.-B3.2., C2, D2-D9, G2-G3, et H1-H2). Cette méthode devrait faciliter le génotypage rapide d'un grand nombre de souches sauvages dans un plus grand nombre de laboratoires. L'application de cette technique à des souches sauvages obtenues lors d'épidémies au Vietnam et en Russie a montré qu'elle peut facilement être adaptée à des situations particulières ou à de nouveaux variants du virus.

RÉFÉRENCES

Atabani, S. F., A. A. Byrnes, A. Jaye, I. M. Kidd, A. F. Magnusen, H. Whittle and C. L. Karp (2001). "Natural measles causes prolonged suppression of interleukin-12 production." J Infect Dis 184(1): 1-9.

Banerjee, A. K. (1987). "Transcription and replication of rhabdoviruses." Microbiol Rev 51(1): 66-87.

Bech, V. (1959). "Studies on the development of complement fixing antibodies in measles patients. Observations during a measles epidemic in Greenland." J Immunol 83: 267-75.

Bellini, W. J., J. S. Rota and P. A. Rota (1994). "Virology of measles virus." J Infect Dis 170 Suppl 1: S15-23.

Belyakov, I. M., B. Moss, W. Strober and J. A. Berzofsky (1999). "Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity." Proc Natl Acad Sci U S A 96(8): 4512-7.

Bohn, W., G. Rutter, H. Hohenberg, K. Mannweiler and P. Nobis (1986). "Involvement of actin filaments in budding of measles virus: studies on cytoskeletons of infected cells." Virology 149(1): 91-106.

Bouche, F. B., O. T. Ertl and C. P. Muller (2002). "Neutralizing B cell response in measles." Viral Immunol 15(3): 451-71.

Caceres, V. M., P. M. Strebel and R. W. Sutter (2000). "Factors determining prevalence of maternal antibody to measles virus throughout infancy: a review." Clin Infect Dis 31(1): 110-9.

Cattaneo, R., G. Rebmann, K. Baczko, V. ter Meulen and M. A. Billeter (1987). "Altered ratios of measles virus transcripts in diseased human brains." Virology 160(2): 523-6.

CDC - Centers for Disease Control and Prevention (1967). "Recommendation of the Public Health Service Advisory Committee on Immunization Practices. Measles vaccines." Morbid Mortal Wkly Rep 16: 269-271.

CDC - Centers for Disease Control and Prevention (2006). "Progress in reducing global measles deaths, 1999-2004." MMWR Morb Mortal Wkly Rep 55(9): 247-9.

Chen, R. T., L. E. Markowitz, P. Albrecht, J. A. Stewart, L. M. Mofenson, S. R. Preblud and W. A. Orenstein (1990). "Measles antibody: reevaluation of protective titers." J Infect Dis 162(5): 1036-42.

Christenson, B. and M. Bottiger (1994). "Measles antibody: comparison of long-term vaccination titres, early vaccination titres and naturally acquired immunity to and booster effects on the measles virus." Vaccine 12(2): 129-33.

Cohen, B. J., R. P. Parry, D. Doblas, D. Samuel, L. Warrener, N. Andrews and D. Brown (2005). "Measles immunity testing: Comparison of two measles IgG ELISAs with plaque reduction neutralisation assay." J Virol Methods.

Cutts, F. (1990). "Measles vaccination in early infancy and persistence of antibody." Lancet 335(8694): 913.

Cutts, F. T., M. Grabowsky and L. E. Markowitz (1995). "The effect of dose and strain of live attenuated measles vaccines on serological responses in young infants." Biologicals 23(1): 95-106.

Cutts, F. T., P. G. Smith, S. Colombo, G. Mann, A. Ascherio and A. C. Soares (1990). "Field evaluation of measles vaccine efficacy in Mozambique." Am J Epidemiol 131(2): 349-55.

Damien, B., S. Huiss, F. Schneider and C. P. Muller (1998). "Estimated susceptibility to asymptomatic secondary immune response against measles in late convalescent and vaccinated persons." J Med Virol 56(1): 85-90.

Davidkin, I. and M. Valle (1998). "Vaccine-induced measles virus antibodies after two doses of combined measles, mumps and rubella vaccine: a 12-year follow-up in two cohorts." Vaccine 16(20): 2052-7.

De Swart, R. L., T. C. Harder, P. S. Ross, H. W. Vos and A. D. Osterhaus (1995). "Morbilliviruses and morbillivirus diseases of marine mammals." Infect Agents Dis 4(3): 125-30.

De Swart, R. L., P. M. Wertheim-van Dillen, R. S. van Binnendijk, C. P. Muller, J. Frenkel and A. D. Osterhaus (2000). "Measles in a Dutch

hospital introduced by an immuno-compromised infant from Indonesia infected with a new virus genotype [letter]." Lancet 355(9199): 201-2.

De Swart, R. L., S. Yuksel and A. D. Osterhaus (2005). "Relative contributions of measles virus hemagglutinin- and fusion protein-specific serum antibodies to virus neutralization." J Virol 79(17): 11547-51.

Dilraj, A., F. T. Cutts, J. F. de Castro, J. G. Wheeler, D. Brown, C. Roth, H. M. Coovadia and J. V. Bennett (2000). "Response to different measles vaccine strains given by aerosol and subcutaneous routes to schoolchildren: a randomised trial." Lancet 355(9206): 798-803.

Dorig, R. E., A. Marcil, A. Chopra and C. D. Richardson (1993). "The human CD46 molecule is a receptor for measles virus (Edmonston strain)." Cell 75(2): 295-305.

El Mubarak, H. S., S. Yuksel, O. M. Mustafa, S. A. Ibrahim, A. D. Osterhaus and R. L. de Swart (2004). "Surveillance of measles in the Sudan using filter paper blood samples." J Med Virol 73(4): 624-30.

Enders, J. F. and T. C. Peebles (1954). "Propagation in tissue cultures of cytopathogenic agents from patients with measles." Proc Soc Exp Biol Med 86(2): 277-86.

Featherstone, D., D. Brown and R. Sanders (2003). "Development of the Global Measles Laboratory Network." J Infect Dis 187 Suppl 1: S264-9.

Fugier-Vivier, I., C. Servet-Delprat, P. Rivailler, M. C. Rissoan, Y. J. Liu and C. Rabourdin-Combe (1997). "Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells." J Exp Med 186(6): 813-23.

Gans, H., L. Yasukawa, M. Rinki, R. DeHovitz, B. Forghani, J. Beeler, S. Audet, Y. Maldonado and A. M. Arvin (2001). "Immune responses to measles and mumps vaccination of infants at 6, 9, and 12 months." J Infect Dis 184(7): 817-26. Epub 2001 Aug 22.

Graves, M., D. E. Griffin, R. T. Johnson, R. L. Hirsch, I. L. de Soriano, S. Roedenbeck and A. Vaisberg (1984). "Development of antibody to

measles virus polypeptides during complicated and uncomplicated measles virus infections." J Virol 49(2): 409-12.

Griffin, D. (2001). Measles Virus. Philadelphia, Lippincott Williams &Wilkins.

Griffin, D. E. (1995). "Immune responses during measles virus infection." Curr Top Microbiol Immunol 191: 117-34.

Griffin, D. E. and B. J. Ward (1993). "Differential CD4 T cell activation in measles." J Infect Dis 168(2): 275-81.

Guris, D., J. McCready, J. C. Watson, W. L. Atkinson, J. L. Heath, W. J. Bellini and A. Polloi (1996). "Measles vaccine effectiveness and duration of vaccine-induced immunity in the absence of boosting from exposure to measles virus." Pediatr Infect Dis J 15(12): 1082-6.

Gustafson, T. L., A. W. Lievens, P. A. Brunell, R. G. Moellenberg, C. M. Buttery and L. M. Sehulster (1987). "Measles outbreak in a fully immunized secondary-school population." N Engl J Med 316(13): 771-4.

Halsey, N. A., R. Boulos, F. Mode, J. Andre, L. Bowman, R. G. Yaeger, S. Toureau, J. Rohde and C. Boulos (1985). "Response to measles vaccine in Haitian infants 6 to 12 months old. Influence of maternal antibodies, malnutrition, and concurrent illnesses." N Engl J Med 313(9): 544-9.

Hanses, F., A. T. Truong, W. Ammerlaan, O. Ikusika, F. Adu, A. O. Oyefolu, S. A. Omilabu and C. P. Muller (1999). "Molecular epidemiology of Nigerian and Ghanaian measles virus isolates reveals a genotype circulating widely in western and central Africa." Journal of General Virology 80(Pt 4): 871-7.

Hanses, F., R. van Binnendijk, W. Ammerlaan, A. T. Truong, L. de Rond, F. Schneider and M. C. P. (2000). "Genetic variability of measles viruses circulating in the Benelux." Archives of Virology 145: 541-51.

Hartter, H. K., R. L. de Swart, F. Hanses, H. W. Vos, F. B. Bouche, A. D. Osterhaus, F. Schneider and C. P. Muller (2000). "Evaluation of different measles IgG assays based on recombinant proteins using a panel of low-titre sera." J Virol Methods 84(2): 191-200.

Hartter, H. K., O. I. Oyedele, K. Dietz, S. Kreis, J. P. Hoffman and C. P. Muller (2000). "Placental transfer and decay of maternally acquired antimeasles antibodies in Nigerian children." Pediatr Infect Dis J 19(7): 635-41.

Hirano, A., A. H. Wang, A. F. Gombart and T. C. Wong (1992). "The matrix proteins of neurovirulent subacute sclerosing panencephalitis virus and its acute measles virus progenitor are functionally different." Proc Natl Acad Sci U S A 89(18): 8745-9.

Hirose, M., Y. Hidaka, C. Miyazaki, K. Ueda and H. Yoshikawa (1997). "Five cases of measles secondary vaccine failure with confirmed seroconversion after live measles vaccination." Scand J Infect Dis 29(2): 187-90.

Holt, E. A., L. H. Moulton, G. K. Siberry and N. A. Halsey (1993). "Differential mortality by measles vaccine titer and sex." J Infect Dis 168(5): 1087-96.

Huiss, S., B. Damien, F. Schneider and C. P. Muller (1997). "Characteristics of asymptomatic secondary immune responses to measles virus in late convalescent donors." Clin Exp Immunol 109(3): 416-20.

Jacobson, R. M., G. A. Poland, R. A. Vierkant, V. S. Pankratz, D. J. Schaid, S. J. Jacobsen, J. S. Sauver and S. B. Moore (2003). "The association of class I HLA alleles and antibody levels after a single dose of measles vaccine." Hum Immunol 64(1): 103-9.

Janeway, C. A. (1945). "Use of consentrated human serum g-globulin in the prevention and attenuation of measles." Bullletin NY Acad. Med. 21: 202-222.

Kaplan, L. J., R. S. Daum, M. Smaron and C. A. McCarthy (1992). "Severe measles in immunocompromised patients." Jama 267(9): 1237-41.

Karp, C. L., M. Wysocka, L. M. Wahl, J. M. Ahearn, P. J. Cuomo, B. Sherry, G. Trinchieri and D. E. Griffin (1996). "Mechanism of suppression of cell-mediated immunity by measles virus." Science 273(5272): 228-31.

Katayama, Y., K. Shibahara, T. Kohama, M. Homma and H. Hotta (1997). "Molecular epidemiology and changing distribution of genotypes of measles virus field strains in Japan." J Clin Microbiol 35(10): 2651-3.

Kennedy, S. (1998). "Morbillivirus infections in aquatic mammals." J Comp Pathol 119(3): 201-25.

Kingston, R. L., D. J. Hamel, L. S. Gay, F. W. Dahlquist and B. W. Matthews (2004). "Structural basis for the attachment of a paramyxoviral polymerase to its template." Proc Natl Acad Sci U S A 101(22): 8301-6.

Kobune, F., H. Sakata and A. Sugiura (1990). "Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus." J Virol 64(2): 700-5.

Koplik, H. (1896). "The diagnosis of the invasion of measles from a study of exanthema as it appears on the buccal mucous membrane." Arch Pediatr 12: 918-920.

Kremer, J. R. and C. P. Muller (2005). "Evaluation of commercial assay detecting specific immunoglobulin g in oral fluid for determining measles immunity in vaccinees." Clin Diagn Lab Immunol 12(5): 668-70.

Krugman, S. (1983). "Further-attenuated measles vaccine: characteristics and use." Rev Infect Dis 5(3): 477-81.

Lee, M. S., L. J. Chien, Y. Y. Yueh and C. F. Lu (2001). "Measles seroepidemiology and decay rate of vaccine-induced measles IgG titers in Taiwan, 1995-1997." Vaccine 19(32): 4644-51.

Liston, P., C. DiFlumeri and D. J. Briedis (1995). "Protein interactions entered into by the measles virus P, V, and C proteins." Virus Res 38(2-3): 241-59.

Markowitz, L. E., S. R. Preblud, W. A. Orenstein, E. Z. Rovira, N. C. Adams, C. E. Hawkins and A. R. Hinman (1989). "Patterns of transmission in measles outbreaks in the United States, 1985-1986." N Engl J Med 320(2): 75-81.

Mathias, R. G., W. G. Meekison, T. A. Arcand and M. T. Schechter (1989). "The role of secondary vaccine failures in measles outbreaks." Am J Public Health 79(4): 475-8.

McNeill, W. (1976). Plagues and Peoples. Garden City, NY.

Moss, W. J., F. Cutts and D. E. Griffin (1999). "Implications of the human immunodeficiency virus epidemic for control and eradication of measles." Clin Infect Dis 29(1): 106-12.

Mossong, J. and C. P. Muller (2000). "Estimation of the basic reproduction number of measles during an outbreak in a partially vaccinated population." Epidemiol Infect 124(2): 273-8.

Mossong, J., C. J. O'Callaghan and S. Ratnam (2000). "Modelling antibody response to measles vaccine and subsequent waning of immunity in a low exposure population." Vaccine 19(4-5): 523-9.

Mulders, M. N., Y. K. Nebie, F. Fack, T. Kapitanyuk, O. Sanou, D. C. Valea, J. J. Muyembe-Tamfum, W. Ammerlaan and C. P. Muller (2003). "Limited diversity of measles field isolates after a national immunization day in Burkina Faso: progress from endemic to epidemic transmission?" J Infect Dis 187 Suppl 1: S277-82.

Mulders, M. N., A. T. Truong and C. P. Muller (2001). "Monitoring of measles elimination using molecular epidemiology." Vaccine 19(17-19): 2245-9.

Muller, C. P. (2001). "Measles elimination: old and new challenges?" Vaccine 19(17-19): 2258-61.

Muller, C. P., S. Huiss and F. Schneider (1996). "Secondary immune responses in parents of children with recent measles." Lancet 348(9038): 1379-80.

Muller, C. P. and M. N. Mulders (2002). Molecular epidemiology of measles virus. The Molecular Epidemiology of Human Viruses. T. Leitner. Boston, Kluwer Academic Publishers: 237-272.

Nokes, D. J., F. Enquselassie, W. Nigatu, A. J. Vyse, B. J. Cohen, D. W. Brown and F. T. Cutts (2001). "Has oral fluid the potential to replace serum for the evaluation of population immunity levels? A study of measles, rubella and hepatitis B in rural Ethiopia." Bull World Health Organ 79(7): 588-95.

Norrby, E., J. Kovamees, M. Blixenkrone-Moller, B. Sharma and C. Orvell (1992). "Humanized animal viruses with special reference to the primate adaptation of morbillivirus." Vet Microbiol 33(1-4): 275-86.

Outlaw, M. C. and C. R. Pringle (1995). "Sequence variation within an outbreak of measles virus in the Coventry area during spring/summer 1993." Virus Res 39(1): 3-11.

Panum, P. (1939). "Observations made during the epidemic of measles on the Faroe Islands in the year 1849."

Pirquet, C. v. (1908). "Verhalten der kutanen tuberkulin-reaktion während der Masern." Deutsch Med Wochenschr 34: 1297-1300.

Ray, J. and R. S. Fujinami (1987). "Characterization of in vitro transcription and transcriptional products of measles virus." J Virol 61(11): 3381-7.

Reyes, M. A., M. F. de Borrero, J. Roa, G. Bergonzoli and N. G. Saravia (1987). "Measles vaccine failure after documented seroconversion." Pediatr Infect Dis J 6(9): 848-51.

Rhazes, A.-B. (1748). Treatise on the Smallpox and Measles. London.

Riddell, M. A., J. S. Rota and P. A. Rota (2005). "Review of the temporal and geographical distribution of measles virus genotypes in the prevaccine and postvaccine eras." Virol J 2: 87.

Riley, E. C., G. Murphy and R. L. Riley (1978). "Airborne spread of measles in a suburban elementary school." Am J Epidemiol 107(5): 421-32.

Rima, B. K., J. A. Earle, K. Baczko, V. ter Meulen, U. G. Liebert, C. Carstens, J. Carabana, M. Caballero, M. L. Celma and R. Fernandez-Munoz (1997). "Sequence divergence of measles virus haemagglutinin during natural evolution and adaptation to cell culture." J Gen Virol 78(Pt 1): 97-106.

Rota, J. S., J. L. Heath, P. A. Rota, G. E. King, M. L. Celma, J. Carabana, R. Fernandez-Munoz, D. Brown, L. Jin and W. J. Bellini (1996). "Molecular epidemiology of measles virus: identification of pathways of transmission and implications for measles elimination." Journal of Infectious Diseases 173(1): 32-7.

Rota, J. S., Z. D. Wang, P. A. Rota and W. J. Bellini (1994). "Comparison of sequences of the H, F, and N coding genes of measles virus vaccine strains." Virus Research 31(3): 317-30.

Rota, P. A., S. L. Liffick, J. S. Rota, R. S. Katz, S. Redd, M. Papania and W. J. Bellini (2001). "Genotypes of Measles Viruses Associated with Outbreaks and Sporadic Cases in the United States: 1997-2000." in preparation.

Ruckle, G. and K. D. Rogers (1957). "Studies with measles virus. II. Isolation of virus and immunologic studies in persons who have had the natural disease." J Immunol 78(5): 341-55.

Schneider-Schaulies, S., U. G. Liebert, K. Baczko, R. Cattaneo, M. Billeter and V. ter Meulen (1989). "Restriction of measles virus gene expression in acute and subacute encephalitis of Lewis rats." Virology 171(2): 525-34.

Schneider-Schaulies, S., S. Niewiesk, J. Schneider-Schaulies and V. ter Meulen (2001). "Measles virus induced immunosuppression: targets and effector mechanisms." Curr Mol Med 1(2): 163-81.

Schnorr, J. J., M. Seufert, J. Schlender, J. Borst, I. C. Johnston, V. ter Meulen and S. Schneider-Schaulies (1997). "Cell cycle arrest rather than apoptosis is associated with measles virus contact-mediated immunosuppression in vitro." J Gen Virol 78 (Pt 12): 3217-26.

Schrag, S. J., P. A. Rota and W. J. Bellini (1999). "Spontaneous mutation rate of measles virus: direct estimation based on mutations conferring monoclonal antibody resistance." J Virol 73(1): 51-4.

Sherman, F. E. and G. Ruckle (1958). "In vivo and in vitro cellular changes specific for measles." AMA Arch Pathol 65(6): 587-99.

Sissons, J. G., S. D. Colby, W. O. Harrison and M. B. Oldstone (1985). "Cytotoxic lymphocytes generated in vivo with acute measles virus infection." Clin Immunol Immunopathol 34(1): 60-8.

Stittelaar, K. J., R. L. de Swart and A. D. Osterhaus (2002). "Vaccination against measles: a neverending story." Expert Rev Vaccines 1(2): 151-9.

Tamashiro, V. G., H. H. Perez and D. E. Griffin (1987). "Prospective study of the magnitude and duration of changes in tuberculin reactivity during uncomplicated and complicated measles." Pediatr Infect Dis J 6(5): 451-4.

Tatsuo, H., N. Ono, K. Tanaka and Y. Yanagi (2000). "SLAM (CDw150) is a cellular receptor for measles virus." Nature 406(6798): 893-7.

van Binnendijk, R. S., S. van den Hof, H. van den Kerkhof, R. H. Kohl, F. Woonink, G. A. Berbers, M. A. Conyn-van Spaendonck and T. G. Kimman (2003). "Evaluation of serological and virological tests in the diagnosis of clinical and subclinical measles virus infections during an outbreak of measles in The Netherlands." J Infect Dis 188(6): 898-903.

Waku Kouomou, D. and T. F. Wild (2002). "Adaptation of Wild-Type Measles Virus to Tissue Culture." J Virol 76(3): 1505-1509.

Whelan, S. P., J. N. Barr and G. W. Wertz (2004). "Transcription and replication of nonsegmented negative-strand RNA viruses." Curr Top Microbiol Immunol 283: 61-119.

Whittle, H. C., P. Aaby, B. Samb, H. Jensen, J. Bennett and F. Simondon (1999). "Effect of subclinical infection on maintaining immunity against measles in vaccinated children in West Africa." Lancet 353(9147): 98-102.

WHO - World Health Organization (1992). "Measles control in the 1990s: plan of action for global measles control." (unpublished document WHO/EPI/GEN/92.3; available from Vaccines and Biologicals, World Health Organization, 1211 Geneva 27, Switzerland).

WHO - World Health Organization (1998). "Expanded Programme on Immunization (EPI). Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses." Weekly Epidemiological Record 73(35): 265-9.

WHO - World Health Organization (1999a). "Guidelines for epidemic preparedness and response to measles outbreaks." (unpublished document WHO/CDS/CSR/ISR/99.1; available from Communicable Disease Surveillance and Response,World Health Organization, 1211 Geneva 27, Switzerland).

WHO - World Health Organization (1999b). "The world health report 1999: making a difference." World Health Organization Geneva.

WHO - World Health Organization (2005). "New genotype of measles virus and update on global distribution of measles genotypes." Wkly Epidemiol Rec 80(40): 347-51.

WHO - World Health Organization (2006). "Manual for the laboratory diagnosis of measles and rubella infection." World Health Organization, Geneva.

WHO/UNICEF (2001). "Measles: mortality reduction and regional elimination. Strategic plan 2001-2005." World Health Organization, Geneva.

Wild, T. F., E. Malvoisin and R. Buckland (1991). "Measles virus: both the haemagglutinin and fusion glycoproteins are required for fusion." J Gen Virol 72 (Pt 2): 439-42.

Xu, W., A. Tamin, J. S. Rota, L. Zhang, W. J. Bellini and P. A. Rota (1998). "New genetic group of measles virus isolated in the People's Republic of China." Virus Research 54(2): 147-56.

Yanagi, Y. (2001). "The cellular receptor for measles virus--elusive no more." Rev Med Virol 11(3): 149-56.

RÉSUMÉ : L'épidémiologie moléculaire du virus de la rougeole ainsi que les études d'immunité humorale se sont avérées être des outils majeurs pour faciliter la surveillance et le contrôle de la rougeole. Nous avons suivi l'évolution de l'immunité humorale après vaccination et après convalescence dans des études longitudinales. Chez les personnes vaccinées nous avons observé une perte d'anticorps accélérée lorsque les taux de départ étaient relativement élevés, alors que les taux relativement bas restaient plus stables. L'analyse des taux d'anticorps chez des convalescents réexposés à une souche sauvage du virus a montré que la réexposition peut induire une stabilisation prolongée des taux d'anticorps. La détection d'anticorps dans du fluide buccal est considéré comme alternative au sérum. Notre évaluation d'une méthode ELISA commercialisée à ces fins a cependant montré que la sensibilité de détection était insuffisante lorsque les taux d'anticorps dans le sérum étaient bas. Afin de faciliter le génotypage du virus sur un grand nombre d'échantillons et de palier à l'absence de facilités de séquençage, nous avons développé une méthode simplifiée pour faire la distinction entre les clades et génotypes du virus de la rougeole actifs. Cette méthode est basée sur 6 PCR multiplex différentes qui produisent des fragments PCR de longueur spécifique pour chaque clade ou génotype. La méthode a été développée en utilisant des isolats viraux appartenant aux différents génotypes et évaluée sur des échantillons cliniques prélevés auprès de patients atteints de la rougeole.

SURVEILLANCE ET CONTRÔLE DE LA ROUGEOLE: CARACTÉRISATION DU VIRUS ET DE L'IMMUNITÉ HUMORALE

ABSTRACT: Molecular epidemiology of measles virus as well as the evaluation of humoral immunity have proven to facilitate measles surveillance and control. We studied the evolution of humoral immunity against measles after vaccination and after convalescence in longitudinal studies. Accelerated antibody decay rates were found among vaccinees with high antibody levels at the beginning of the study period, whereas low antibody levels were relatively stable. Re-exposure to wild-type virus induced a longlasting stabilization of antibody levels in convalescents. Since the proportion of vaccinated individuals is continously increasing and wild-type virus circulation is low, regular studies of humoral immunity will be crucial to assess the level of protection of in the corresponding populations. Antibody detection in oral fluid has been promoted as an alternative to serum in the past. Our evaluation of a commercialised ELISA however shows a reduced sensitivity of detection compared to serum when antibody levels were low. In order to facilitate measles virus genotyping on a large number of samples and in absence of sequencing facilities we have developed a simplified genotyping method to distinguish between actif measles virus clades and genotypes. This method is based on 6 different multiplex PCRs, which give PCR products of characteristic sizes for each clade or genotype. The method was developed using virus isolates of the different genotypes and evaluated and further optimized using clinical samples collected from measles patients.

DISCIPLINE : Microbiologie

MOTS-CLÉS : virus de la rougeole, immunuté humorale, vaccination, variabilité génétique, surveillance, contrôle

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