Technical report on the Millennium Cohort Study biomedical data enhancement study of infections and later allergies

Authors

Claire Townsend[1], Mario Cortina-Borja[1], Catherine Peckham[1], David Brown[2], Jon Johnson[3], Heather Joshi[3] and Carol Dezateux[1]

Affiliations

[1] MRC Centre of Epidemiology for Child Health, UCL Institute of Child Health, University College London

[2] HPA Microbiology Services Division, Colindale, Health Protection Agency

[3] Centre for Longitudinal Studies, Institute of Education, University of London

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APPENDICES

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1. INTRODUCTION

The recent increase in childhood asthma and allergic disease throughout developed countries is poorly understood and is of public health concern. At the same time, there has been a marked change in the pattern of common infections in childhood with a decline in their early acquisition. There is evidence to suggest that the increase in prevalence of childhood asthma and allergic disease may be due to changes in standard of living, family structure and size, and childcare, all of which may reduce exposure to infections in the early years. However there have been no studies which have directly tested this theory by measuring infections acquired in early life.

The Millennium Cohort Study (MCS) provided a unique opportunity to document, in a national sample of young children, the pattern of immunity to common childhood infections with different routes of exposure, including varicella zoster virus, Epstein-Barr virus and norovirus. This allows evaluation of the hygiene hypothesis as described by Strachan (1989), which proposes that the increase in asthma and allergic disorders is related to a reduction in the early acquisition of infections at a time when the immune system is still developing. The longitudinal nature of this cohort makes it possible to examine both current and subsequent development of allergic disorders in the context of objective information about both early infections and a wide range of social, family and environmental factors.

New laboratory techniques, based on oral fluid obtained from cohort children, were used to determine how many three year old children had been exposed to three common infections, which may have non-specific symptomatology, selected to represent different routes of transmission. Details of asthma and allergic disease, a planned component of the health information which was sought from cohort members at age three and at future follow ups, is being used to examine the relation between early infection and social and medical factors, including the onset of asthma and allergic disease by school age.

Objective measures of infection in young children are essential in epidemiological studies of acquisition of infections in early life, but blood sampling is not feasible in large-scale population-based studies at this age. However, as many common childhood infections are asymptomatic, or of non-specific symptomatology objective measures are needed. Oral fluid has been used increasingly as a minimally invasive alternative to blood in seroprevalence studies. It is a non-invasive biological sample, which can be returned by post, making it suitable for large-scale epidemiological studies in children.

Effective methods for the collection, transport and processing of oral fluid samples have been developed and evaluated to ensure optimum sample quality. There has been increasing interest in investigating the relationship between common infections in very early childhood but to date, there is only very limited experience of oral fluid collection to measure such infections objectively within large-scale cohort studies of preschool-aged children.

Results from 12,473 oral fluid samples from singleton children in MCS sweep 2 (MCS2) were returned to the Centre for Longitudinal Studies for deposit in the ESDS data archive. Of these 11,034 formed part of the deposit (see Table 2). The samples were tested at the Health Protection Agency (HPA) for total immunoglobulin G (IgG) content and antibodies to varicella zoster virus (VZV), Epstein-Barr virus (EBV) and norovirus.

2. STUDY DEVELOPMENT

A prospective study was conducted to collect oral fluid samples in the home setting from 3-year-old children enrolled in the MCS. The study population comprised all cohort children participating in the second MCS survey conducted between September 2003 and January 2005. At this second survey, children were interviewed at home by social survey interviewers. These interviewers were experienced in social and biomedical surveys and were given additional training to obtain informed consent and to guide the collection of oral fluid samples from cohort participants, as described below. Translators were made available when neither a resident parent nor other household member aged over 16 years spoke English. At each interview of the four surveys so far, information was collected on a range of demographic, social, and health factors related to the child and their family as described in Hansen (2010).

This is to our knowledge the first large-scale child cohort study in which oral fluid has been collected from preschool-aged children in the home setting, with an explanation from a trained interviewer. We achieved a high response rate, which indicates that using this methodology to collect biological samples from young children is feasible and acceptable. Further details appear in Bartington et al (2009).

3. RESEARCH ETHICS APPROVAL

The South West Multicentre Research Ethics Committee (MREC) granted approval for the first sweep of the MCS interviews. Fieldwork for the second interview and approval for the collection and analysis of oral fluid samples for the presence of antibodies to EBV, VZV and norovirus, was approved by the London MREC and registered with the Research and Development Office at the UCL Institute of Child Health.

4. SAMPLE DESIGN

The MCS is a prospective study of the social, economic and health-related circumstances of British children born in the new century. A stratified clustered survey design was employed to over-represent children living in disadvantaged areas, from ethnic minority groups and the smaller UK countries. The original cohort comprised 18,818 children (18,552 families), living in the United Kingdom at age 9 months when the first survey was conducted, representing a response rate of 72% at the first survey. A detailed description of the sampling design is provided in Plewis (2007).

5. FIELDWORK PROCEDURES

In addition to the main questionnaire and child assessment survey the fieldwork for the second MCS survey included collection of oral fluid from cohort children.

Interviewer training

Interviewers were trained in oral fluid collection by a slide presentation and short video during one of the briefing days prior to commencement of the MCS2 fieldwork (NOP, 2006). The guidance allowed for the sample to be collected by either the interviewer or a parent or caregiver depending on who the child felt most comfortable with. Interviewers were shown how to collect the sample and also how to guide the parent or caregiver to collect the oral fluid sample, and to leave the kit with the mother if the sample was not collected at the time of interview.

Invitation to participate

Families who were eligible for the second MCS survey were posted a letter and four-page study booklet giving information about the second survey a few days before the home interview. This included the "Infections and Allergies Study" leaflet shown in **Appendix A1** inviting them to participate in the oral fluid survey, with a short description of the hygiene hypothesis as the purpose of collecting oral fluid (referred to as "saliva" for simplicity) and the collection method that would be used. The letter and leaflet were translated into ten languages (Arabic, Bengali, Gujarati, Hindi, Kurdish, Punjabi, Somali, Tamil, Turkish, Urdu), with translated versions sent to households identifying one of these as their main household language at the first survey. All families in Wales were sent a study booklet in English and Welsh.

Consent procedure

As part of the consent procedure for the second survey, the interviewer reminded the main responding parent or other carer (usually the mother) in the cohort child's household about the information letter, repeated assurances about confidentiality, and offered to answer any questions arising from the letter and information sheet. The respondent was then asked if they would like to make an appointment for a later interview visit, or if they preferred to start the interview process straight away.

Before beginning any of the actual data collection, the interviewer asked the main parent (or other alternative main respondent) to give formal consent to the process by completing a consent form shown in **Appendix A2**. The consent form consisted of three separate sections – one for the parental interview, one for the cohort child covering the assessments, measurements, oral fluid collection and gathering of administrative data on hospital admissions, and one for the material relating to older siblings, including permission for the collection of statistical data from school records. It was made clear to respondents that they could give permission for some elements and refuse permission for others. Duplicate copies of the consent form were supplied, watermarked "Your Copy" and "Office Copy". Both the interviewer and the respondent signed each copy, and one copy was left with the respondent while the other was sent back to the fieldwork agency. At the time of this study the cohort study leadership team were concerned that parents might think this sample would be used for DNA analysis and that this might impact on cohort retention. In view of

this specific reassurances were required to be given as specified in the "Infection and later allergies" information leaflet for parents (**Appendix A1**): namely parents were informed that no genetic tests would be performed on the samples and that any remaining samples would be destroyed after the specified tests had been carried out. This policy of non-retention reflected a cautious approach to the first biological samples being requested of the cohort families, and concerns that good relations with them might be jeopardized if they thought the material might be put to other uses.

Translation and interpreter support

The study funding prohibited formal translation of the extensive lengthy computerised questionnaire instrument into different languages, so all translation, whether by household members or outside translators, was carried out verbally (apart from the Welsh cognitive assessments). Details are provided elsewhere (NOP, 2006).

Oral fluid sample collection

Interviewers were requested to perform the oral fluid sample collection at any time during the interview when the child was ready and would cooperate. The interviewers were informed about oral fluid collection methods using a purpose-made video presentation, and explained to participating mothers how to collect a sample of oral fluid from their child using the Oracol oral fluid collection device (Malvern Medical Developments Ltd, Worcester, UK). This device, which has been widely used in the UK, was selected for its acceptability for use with children and for its high oral fluid sample quality. The device consists of a polystyrene foam swab at the end of a plastic stick. The absorbent foam swab included with this device was gently rubbed over the child's gums for one minute by the mother or the interviewer (if the child preferred) using an action similar to that when brushing teeth. The sample collected in this way comprises oral fluid containing crevicular fluid which contains antibody transudate from the gum capillaries. If the child did not like the sponge being rubbed on the gum margin, mothers were asked to leave the sponge in the space between the cheek and the bottom teeth and let it soak up the saliva for one minute. If the child did not wish to provide a sample at the time of the interview, interviewers were instructed to ask the parent or caregiver to collect the sample at a later date using the same procedure, and issued the sample collection tube, and postage paid padded envelope.
This sample collection procedure has been shown to be acceptable to children and to cause minimal discomfort (Nokes et al, 1998).

Oral fluid sample transport

After collection of oral fluid, the device was sealed in the supplied plastic tube, inserted into a larger tube labelled with a barcode denoting the household identification number and the date and time of sample, and posted using a pre-addressed reply-paid padded envelope to the laboratory. Barcoded labels were provided by the fieldwork agency, and to ensure confidentiality, no names were printed on the labels. However, due to the use of a

household-specific rather than a child-specific identifier, twins and triplets were not uniquely identified by the bar-coded label.

Interviewers, or parents if the sample was collected after the interview, were instructed to record the date and time of sample collection on the protective tube. A designated courier or the Royal Mail postal service was used for transport of samples to the HPA Microbiology Services Division.

6. PROCESSING AND ANALYSIS OF SAMPLES

Laboratory methods

Processing and storage of samples

Upon arrival at the HPA, oral fluid samples had their barcode scanned to generate a date of sample receipt. The MCS family identification number encoded in the barcode was removed at this stage and replaced by a laboratory identification number, the MOLIS ('Modular Open Laboratory Information System') number. All further analyses conducted at the HPA used the MOLIS number for sample identification.

Following arrival at the HPA laboratory, samples were extracted from the sponge swab by agitation of the swab for 30 seconds in 1 mL transport medium (Phosphate buffered saline pH 7.2 with 10% fetal calf serum 0.2% Tween 20, 0.5% gentamicin, and 0.2% fungizone) followed by centrifugation. Oral fluid samples were then divided into four aliquots before freezing at - 20°C. **Appendix B1** contains the protocol to extract oral fluid samples from foam swabs.

IgG oral fluid assay

The assay used to test oral fluid samples for total IgG was a TRFIA assay, using conjugated human IgG antibody (DELFIA). A dilution series (2.5 mg/L to 0.039 mg/L) of human IgG was run on each sample plate. See **Appendix B2** for further details on the assay protocol.

VZV oral fluid assay

A new VZV assay was developed by Maple and colleagues (2009) at the HPA Microbiology Services Division, based upon a TRFIA format, adapted from a published serum assay. The serum assay was adapted for oral fluid using serial dilutions and testing of a panel of paired serum and oral fluid samples. The assay protocol followed is in **Appendix B3**.

EBV oral fluid assay

The TRFIA assay used for detection of antibodies to EBV was developed at the HPA Microbiology Services Division, and is based on the work of Sheppard and colleagues (2001). The assay detects antibodies against the EBV Capsid Antigen (EBV-CA). The oral fluid assay used the TRFIA format and was based on commercially available EBV Capsid antigen. This provided a quantitative assay for human IgG antibodies against EBV-CA in serum. In this process the wells are coated with EBV-CA and diluted patient samples are placed within them. Specific IgG antibodies (also IgA and IgM) bind to the antigens; a second incubation is then carried out using labelled anti-human IgG, producing fluorescent counts. The serum

assay was optimised for oral fluid at the HPA using serial dilutions and comparison of concordance of results between the serum and oral fluid samples, using a matched oral fluid/serum sample panel. The assay protocol followed is in **Appendix B4**.

Norovirus oral fluid assay
An in-house TRFIA assay for norovirus was developed at the HPA based on the assay described in Gray et al (1993). The assay protocol followed is in **Appendix B5**.

Adenovirus assay development
It was initially planned that samples would also be tested for antibodies to an adenovirus. However, the assay failed during the developmental phase of the project, and it was therefore not possible to test samples for exposure to this type of virus.

Determining infection status using oral fluid assay results

Ideally, latent class mixture models should be employed to determine the probability of being infected while adjusting for the influence of relevant risk factors and measures of sample quality (for example, see Hardelid et al, 2009). At the time of writing this report, the authors did not have access to the full range of variables needed to conduct these analyses. The deposited data and this report will be updated once this work has been completed.

For the purposes of this report we have provided in the text below some proposed cut-offs for each specific assay. These figures have been provided by colleagues at the HPA, with a strong caveat for users, who are cautioned against regarding them as a definitive approach to determining infection status. Hence they can be only interpreted as providing a very crude and simplistic indication of infection status.

Crude cut-off values for each of the assays are suggested as follows:

VZV – 0.277 mIU (International Units)/mL (Chris Maple, HPA, personal communication, and see reference Maple et al, 2009).

EBV – 4.74 AU (Arbitrary Units) (David Brown, HPA, personal communication).

Norovirus – 1.61 RU (Relative Units) (David Brown, HPA, personal communication).

Data entry and processing

Assay results from the testing of MCS samples were manually entered from the printout obtained from the fluorimeter, with count and unit values provided. The plate identification number and test date were also entered. Later in the study these could be downloaded directly on an Excel spreadsheet. A master spreadsheet was compiled containing all results from the assay testing. Oral fluid assay data were collated in Excel and imported into Stata by the researchers for all further linkage and analyses.

7. RESPONSE RATES

A total of 12,943 oral fluid samples were recorded; 119 were excluded due to missing, incorrect or non-unique identifiers.

A further 351 samples were from twins or triplets; individual children could not be distinguished as only the household (not child) identifier had been printed on the barcoded label (Table 1).

Table 1 – Distribution of 12824 samples according to singleton/multiple birth (excluding 119 with missing, incorrect or non-unique identifiers)

	Order of samples			Total
	First*(n)	Second (n)	Third (n)	
Singletons	12,473	-	-	12,473
Twins	166	162	-	328
Triplets	8	8	7	23

Among families of singleton children in MCS2, 81.1% (12,473/15,382) returned a sample. Of these, written consent was obtained for 88.5% (11,034/12,473) (Table 2). The first two categories of consent in Table 2 were considered as evidence of valid consent, while for categories 3 to 5, there was no clear evidence of valid consent despite sample receipt.

Table 2 – Evidence of consent in relation to samples received (singletons only)*

	***n* (%)**
1. Consented, with parent & interviewer signatures	11,027 (88.4%)
2. Consented, with parent signature only	7 (0.1%)
3. Consented, with interviewer signature only	9 (0.1%)
4. Consented, no signatures	15 (0.1%)
5. Consent box not ticked	1,415 (11.4%)
Total	12,473 (100%)

Response rates and associated maternal and child factors, including those relevant to allergic disorders or asthma, were explored in a paper published by Bartington et al (2009); note that this analysis was based on sample receipt and not consent. In the published article, Bartington et al excluded 608 children recruited at the second MCS sweep as the analysis required predictor variables collected at age 9 months in the first survey, leaving 11,883 children (95% of those with oral fluid samples, and 81% of all singletons with data available at MCS1 and MCS2). Over 98% of the 11,883 samples were suitable for assay

testing. Children whose mothers were of Black Caribbean ethnicity or who lived in non-English speaking households were less likely to provide a sample. Mothers reporting a history of asthma were more likely to return a sample from their child. Further details are reported in the publication.

8. CODING AND EDITING

Data cleaning – sample collection and receipt dates

The interval between sample collection and sample receipt dates was calculated, and inconsistencies were investigated by cross-checking with interview date. Corrections were made where sample year, month and/or day was deemed to be incorrect. In total, 203 corrections were made to dates of sample or receipt.

Sample quality - IgG concentration

IgG concentration has been shown in other studies to provide a reliable measure of oral fluid sample quality. As discussed previously, latent class mixture models are the appropriate method for determining the distribution of quality. Here we suggest a crude indication of quality based on a cut-off value of 2mg/L (personal communication, Farah Aladin, HPA). This is based on the following work and assumptions. Samples from cohort children with and without a reported history of chicken-pox were tested for total IgG, using the optimised assay. The count values from the assay were converted into mg/L units, as a serially diluted standard of known total IgG concentrations was included in each assay performed. Histograms were generated using the data from each population and overlaid on the same axis (log10 mg/L vs frequency). The point at which the populations of VZV-positive and VZV-negative intersected was 2.5 mg/L. However, in recognition of the fact that up to 10% of individuals with a negative VZV history could be misclassified, a more conservative cut-off of 2 mg/L is suggested.

This provides a relatively crude measure of quality. The prevalence of samples with IgG less than 2 mg/L in the MCS dataset is approximately 45%. The most likely explanation for low concentrations of IgG is that insufficient oral fluid has been collected on the device. Although the interviewer/parent/carer was guided to rub the Oracol sponge on the child's gums for one minute, in the absence of a timer device or visual indicator to reveal the volume of oral fluid collected onto the device it is likely that there was variation in the time taken to collect the sample, the sample volume or both and that this is reflected in the oral fluid IgG concentrations achieved after elution of the sample. In future studies improvements of sample quality may be obtained by using a collection device with a visual indicator of sample volume quality. Latent class mixture models can be used to condition on sample quality when determining seroprevalence of infection, so it is not suggested that samples with total IgG concentrations below this threshold should be omitted from analyses.

9. SEROPREVALENCE

Table 3 summarises the prevalence of prior infection with the specified viruses using the crude threshold values reported above, stratified according to whether the oral fluid sample total IgG is above or below the specified threshold. As can be seen, the seroprevalence is higher in samples with higher IgG concentrations. This report and the data will be updated when analyses using latent class mixture models have been completed.

Table 3

	Seroprevalence of infection		
Virus	**Overall**	**Total IgG <2 mg/L**	**Total IgG ≥2 mg/L)**
VZV (≥0.277 mIU/mL) (*n*=12,414)	33.4%	17.9%	46.8%
EBV (≥4.64 AU) (*n*=12,416)	17.6%	5.1%	28.5%
Norovirus (≥1.61 RU) (*n*=12,395)	22.5%	8.2%	34.8%

10. VARIABLE LIST AND DESCRIPTION

Archived variables are as follows:

Variable name	Variable label	Type
mcsid	Patient identifier	discrete
ebvunits	EBV units (AU/ml)	continuous
norounit	Norovirus units (RU/ml)	continuous
vzvunits	VZV units (mIU/ml)	continuous
iggunits	IgG units (mg/L)	continuous
sampintl	Interval between sample collection & receipt (days)	continuous

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12. CONTACTS

Principal Investigators: Professor Carol Dezateux (c.dezateux@ucl.ac.uk) and Professor Catherine Peckham (c.peckham@ucl.ac.uk)

Researchers: Dr Claire L Townsend (c.townsend@ucl.ac.uk) and Dr Mario Cortina-Borja (m.cortina@ucl.ac.uk)

MRC Centre for Epidemiology of Child Health, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH.

13. HOW TO ACKNOWLEDGE THIS DATA RESOURCE

Users of these data are required to acknowledge their provenance in all published outputs The appropriate form appears in the Bibliographic citation section of the Study information and citation document that accompanies the data set. All cohort data now have DOI references, called Persistent Identifiers, which should be included in the citation.

There should also be an acknowledgement. An appropriate form of words for this would be: "I am grateful to The Centre for Longitudinal Studies, Institute of Education for the use of these data and to the UK Data Archive and Economic and Social Data Service for making them available. However, they bear no responsibility for the analysis or interpretation of these data."

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Appendix A: Information sheet and consent form

A1. Allergies Information Sheet

Infections and later allergies

Do common infections in early childhood protect against later allergies?

Parents information sheet

It has been suggested that common and often unnoticed infections during early childhood may protect children against developing allergic diseases such as asthma and hay fever later in childhood and adult life. To find out whether this is the case we are asking all parents of the Child of the New Century children to help us find out whether their child has had any of these infections in the past.

We would like you to collect some saliva (spit) from your child. The saliva on the gums contains substances that tell us whether your child has had any of these infections before. We can collect the saliva using a gum swab. The gum swab is a sponge designed to be used like a toothbrush. Using it is a simple, painless process, much like brushing your teeth.

Collecting saliva
Saliva is collected by simply rubbing the sponge firmly along the gum at the base of the teeth, until the sponge becomes moist. This takes about one minute.

What happens to the saliva?
The interviewer will send it to the laboratory where the tests are done. Any sample left after testing is completed will be thrown away at the end of the study. No genetic tests will be done.

What happens to the results?
The infections we are measuring are common and often go unnoticed. The laboratory uses bar-coded labels (like the ones used in supermarkets) and has no way of knowing your child's identity. Results will not be given back to individual families or GPs.

Your help would be gratefully appreciated and the results of this work would allow us to make a real contribution to child health.

Project Director: Professor Heather Joshi, OBE
Centre for Longitudinal Studies ▪ Institute of Education ▪ University of London
FREEPOST LON20095 ▪ London ▪ WC1H 0BR

A2. Consent form

Form 2: Consent for the Cohort Child

I (name),_____ give my consent to

_____ (Interviewer)

to carry out the following Measurements: (tick one for each)	Yes	No
a) Knowledge of colours, letters, numbers and counting, sizes, comparisons and shapes,	☐	☐
b) Names of objects	☐	☐
c) Standing height	☐	☐
d) Body weight	☐	☐

of my child (name)_____

I have read the letter of introduction and the information leaflet about the Second Survey of the 'Child of the New Century'. I have discussed any outstanding questions with the interviewer named below and I wish my child named above to participate in the Measurements. I understand that I can stop the interview and the Measurements at any point or decline any part of it, and that all information will be treated in the strictest confidence and used for research purposes only.

☐ **I give my consent to my child providing a sample of saliva (spit).**

I have read the letter of introduction, the 'Infections and Allergies Study' leaflet and the information leaflet about the Second Survey of the 'Child of the New Century'. I have discussed any outstanding questions with the interviewer named below and I wish my child named above to participate in this study. I understand that I can stop the interview and the collection of saliva (spit) at any point or decline any part of it, and that all information will be treated in the strictest confidence and used for research purposes only.

☐ **I give my consent to use of information from National Health Service records on date and reason for hospital admission and treatment for my child from birth to age 7 years in future medical research studies of the causes, diagnosis, treatment or outcome of disease.** I understand that the information obtained by the investigators will be coded and used anonymously for research purposes only, and will not include my name or address. I understand that I may withdraw this consent at any time by contacting the investigators in writing, without giving any reasons.

Signature by Parent

Signed _____ **Date** _____

Countersignature by Interviewer

I confirm that I have explained the nature of the proposed studies to the person named above and have left a copy of the information sheet and this consent form with them for future reference.

Signed _____ **Date** _____

Appendix B: Oral fluid assay laboratory protocols

B1. Extraction of saliva samples from foam swabs

1. Add 1 ml Transport Medium to tube containing swab.

2. Agitate swab manually, or use bench vortexer for 20 seconds to ensure foaming of transport medium.

3. Remove swab from tube using a twisting motion to extract as much liquid as possible from the swab.

4. Invert swab and replace it in the tube so that the pink foam is now at the top of the tube. Replace cap.

5. Centrifuge at 2000 rpm for 5 minutes.

6. Discard swab using forceps.

7. Extracted saliva can now be recovered from the tube, using a pasteur pipette.

8. Store at -20°C prior to testing.

Transport Medium	**100 ml Volume**
10% Foetal Calf Serum	10 ml
0.2% Tween 20 (Sigma)	20 µl
Phosphate Buffered Saline	90 ml
0.5% Gentamicin (50 mg/ml stock)	500 µl
0.2% Fungizone (250 µg/ml stock)	200 µl
red food dye * 50µl	

*The addition of red dye is optional. It is used to help specimen handling in the laboratory; eg, to make it easy to confirm that the sample has been added to the well of an ELISA plate

B2. Total IgG oral fluid assay

1) Coat DELFIA microtitre plate(s) with 100µl/well of 1:4000 Protein A (Sigma) diluted in 0.05M carbonate buffer, pH 9.6 (need 10ml/plate).

2) Cover plate with a plate sealer, and incubate at 4°C overnight in the cold room, in the box provided.

3) The following morning (NB bring wash concentrate and assay buffer to room temp beforehand), wash plate with DELFIA wash buffer (dilute 25x wash concentrate i.e 40ml concentrate in 960ml SDW) using a DELFIA plate washer (4 washes with a 10sec soak cycle – program 04 AFP 2 Inc W2) or other validated washer (Denley Wellwash [Soak:1, Pause:6, Washes:4, Vol:8]) and wash cycle. Blot/tap plate to remove any residual wash solution in wells.

4) Prepare oral fluid specimens by vortexing briefly and then microfuging (3,500rpm for 10mins) and add 100µl 1:10 oral fluid sample diluted in assay buffer, by adding 10µl sample to appropriate well, followed by 90µl assay buffer to assigned wells on the plate.

5) Also run a doubling dilution series (2.5mg/l to 0.039mg/l) of human IgG (Binding Site) using wells A1-G1 of the plate. Also include assay buffer control (well H1).

6) Cover plate with a plate sealer and incubate at 37°C for 2hrs in a moist box.

7) Wash plate (as described above in step 3) and add 100µl 1:2000 europium-conjugated anti-human IgG antibody (DELFIA) e.g. as stock concentration is 50µg/ml, add 20µl antibody to 10ml assay buffer, with 1:50 mouse serum (Sera Laboratories International).

8) Incubate plate(s) at 37°C for 2hrs in a moist box.

9) Wash plate (as described above in step 3) and add 150µl neat enhancement solution, and cover with a plate sealer.

10) Incubate plate whilst shaking on an orbital shaker for 10mins at room temperature in a dark environment.

11) Read plate using the Victor3 fluorimeter and the Total IgG program.

*2.5mg/l dilution should read ~40,000-60,000 counts (with halving values for subsequent dilutions), blank should read ~400-900 counts.

Reagents	**Location**
Plates	3C30
Protein A: Add 5µl to 20ml coating buffer	3C22 (-40^{o}C)
Coating buffer	Cold room
Standard curve	3C22 (-40^{o}C)
Assay buffer: in use	3C22 (4-8^{o}C)
Assay buffer :stock	TRFIA room (EM Suite)
Wash concentrate: in use	3C22 (4-8^{o}C)
Wash concentrate: stock	TRFIA room (EM Suite)
Enhancement solution: in use	3C22 (4-8^{o}C)
Enhancement solution: stock	TRFIA room (EM Suite)
Anti-Hu IgG EU conjugate	3C22 (4-8^{o}C)
Mouse serum	3C22 (-40°C)

Summary of quantities of reagents for 2 plates:

Coating of plates
20ml 0.05M carbonate buffer
5µl Protein A

Addition of samples
100µl standard curve and blank
10µl oral fluid sample (plus 90µl assay buffer)

Addition of conjugate
20ml assay buffer
10µl Europium-conjugated anti-human IgG
400µl mouse serum

Enhancement solution
150µl enhancement solution

B3. VZV oral fluid assay

VIRUS REFERENCE DIVISION

STANDARD OPERATING PROCEDURE

TITLE : TIME-RESOLVED FLUORESCENCE IMMUNOASSAY FOR THE DETECTION OF VZV IgG ANTIBODIES IN SERUM AND ORAL FLUID

SOP NO. V- 5364/02-05 **EFFECTIVE DATE** 15.06.05

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WRITTEN BY : CHRIS MAPLE

AUTHORISED Christopher Gallimore DATE 17.06.05

ISSUED TO COPY NO.

SUMMARY

The detection of VZV IgG antibodies in serum and oral fluid is described.

SAFETY

Wear lab coat and gloves

Refer to COSHH No. VZV Delfia

BioCOSHH: Immunoassays for the detection of VZV antibodies in serum and oral fluid

1.0 CROSS REFERENCE

1.1 QSOP27: Quality assurance in the diagnostic virology and serology laboratory

2.0 EQUIPMENT

2.1 Single channel digital pipette 100-l000ul, 20-200ul and 10-100ul

2.2 Multichannel digital pipette 50-300ul - 2 needed including a dedicated pipette required for europium addition

2.3 Mini Orbital Shaker

2.4 Delfia 1234 Fluorometer

2.5 Delfia 1296-026 Platewash

3.0 REAGENTS

3.1 Delfia microtitre plates 1244-5 5 0 (Perkin Elmer)

3.2 VZV Elisa grade antigen (Binding Site, UK)

3.3 British Standard VZV Antibody 90/690 (NIBSC, UK)

3.4 0.05M Carbonate buffer, pH 9.2-9.6 (Internal)

3.5 Delfia wash buffer 1244-114 (Perkin Elmer)

3.6 Delfia assay buffer 1244-111 (Perkin Elmer)

3.7 Europium labelled anti-human IgG conjugate 1244-330 (Perkin Elmer)

3.8 Delfia enhancement solution 1244-105 (Perkin Elmer)

4.0 PROCEDURE

4.1 The required number of plates are coated overnight with a pre-determined concentration of VZV antigen diluted in carbonate buffer.

4.2 The next day Delfia wash buffer is prepared and the coated plates are washed four times (program 39) using the Delfia Platewash.

4.3 Serum samples are diluted 1:50 in assay buffer ie. 10ul sample to 490ul assay buffer and l00ul added to appropriate wells, in duplicate. Oral fluid samples are microfuged for 10 minutes at 10,000 rpm and l00ul added, undiluted, to appropriate wells.

4.4 A standard curve is run on each plate, for serum plates the concentration series of British Standard VZV antibody is 50 mIU/ml, 25 mIU/ml, 12.5 mIU/ml, 6.25 mIU/ml, 3.12 mIU/ml, 1.56 mIU/ml, 0.78 mIU/ml and 0.39 mIU/ml. For oral fluid plates the concentration series used is 5.0 mIU/ml, 2.5 mIU/ml, 1.25 mIU/ml, 0.6 mIU/ml, 0.3 mIU/ml, 0.15 mIU/ml and 0.07 mIU/ml. 100 ul of each concentration of standard is loaded, in duplicate, into appropriate wells.

4.5 Assay buffer (l00ul) is also loaded, in duplicate, to designated wells to act as a background control.

4.6 Once loaded the plate is sealed. Serum plates are incubated for 2 hours at 37 C in a wet chamber. Oral fluid plates are incubated for 2 hours at room temperature in a wet chamber on a mini orbital shaker set at 100 rpm.

4.7 Following 2 hours incubation the plates are washed four times (see 4.2). Europium labelled anti-human IgG conjugate (lOOul) at a dilution of 1:500 in assay buffer is added to all wells using a multichannel pipette. The plates are not sealed.

4.8 The plates are incubated as described in 4.6; however, serum plates are only incubated for 1 hour.

4.9 The plates are washed as in 4.2.

4.10 150 ul Delfia enhancement solution are added to all wells using a dedicated multichannel pipette. The plates are then incubated for 15 minutes, in the dark, on the mini orbital shaker set at 100 rpm.

4.11 The plates are then read using the 1234 fluorometer. For serum plates Program 9 "VZV TRFIA" is used and for oral fluids Program 10 (Saliva Assay) is used.

4.12 Results should only be accepted if internal quality control is satisfactory following application of the Westgard Rules (+3SD and l0x) to appropriate concentrations of the VZV antibody standard. Background europium counts for the assay buffer controls should not exceed 1500

4.13 For VZV IgG antibody concentrations in sera less than 100 mIU/ml report as "VZV IgG antibody NOT detected". For concentrations in excess of 90 mIU/ml the possibility that the sample contains very low concentrations of antibody needs to be considered and additional comments may be required based on the clinical information available. Advisable to retest specimen.

4.14 For VZV IgG antibody concentrations 100 mIU/ml to 150 mIU/ml report as "Consistent with a low concentration of VZV IgG which may not be detected by some commercial assays". Advisable to retest specimen.

For VZV IgG antibody concentrations in excess of 150 mIU/ml report as “VZV IgG detected consider immune”.

B4. EBV oral fluid assay

1) Coat DELFIA microtitre plate(s) with 100µl/well of 2.0 ug/ml EBV VCA antigen diluted in 0.05M carbonate buffer, pH 9.6 (need 10ml/ plate).
2) Cover plate with a plate sealer, and incubate at 4°C overnight in the cold room, in the box provided.
3) The following morning (NB bring wash concentrate and assay buffer to room temp beforehand), wash plate with DELFIA wash buffer (dilute 25x wash concentrate i.e 40ml concentrate in 960ml SDW) using a DELFIA plate washer (4 washes with a 10sec soak cycle – program 39) or other validated washer (Denley Wellwash) and wash cycle. Blot/tap plate to remove any residual wash solution in wells.
4) Prepare oral fluid specimens by vortexing briefly and then microfuging (3,500 rpm for 10 mins) and add 100µl samples (in duplicate for first 1,000 specimens) to assigned wells on the plate.
5) Also run a dilution series (1/50-1/6400) of EBV positive serum (1395 VCA +, EBNA -) using columns 1 & 2 of plate. Also include assay buffer control (2 wells) and internal quality control* preparation (2 wells).
6) Cover plate with a plate sealer and incubate at 37°C for 2hrs in a moist box.
7) Wash plate (as described above in step 3) and add 100µl 1:500 europium-conjugated anti-human IgG antibody (e.g. as stock concentration is 50µg/ml, add 20µl antibody to 10ml assay buffer).
8) Incubate plate(s) at 37°C for 2hr in a moist box.
9) Wash plate (as described above in step 3) and add 150µl neat enhancement solution.
10) Incubate plate whilst shaking on an orbital shaker for 10mins at room temperature in a dark environment.
11) Read plate using either/both fluorimeters using the standard test programs.

*IQC should be serum diluted in transport medium giving counts of 10,000-15,000.

B5. Norovirus oral fluid assay

1) Coat DELFIA microtitre plate(s) with 100µl/well of 2.0 ug/ml rGV antigen diluted in 0.5M carbonate buffer, pH 9.6 (need 10ml/ plate).
2) Cover plate with a plate sealer, and incubate at 4°C overnight in the cold room, in the box provided.
3) The following morning (NB bring wash concentrate and assay buffer to room temp beforehand), wash plate with DELFIA wash buffer (dilute 25x wash concentrate i.e 40ml concentrate in 960ml SDW) using a DELFIA plate washer (4 washes with a 10sec soak cycle – program 04 AFP 2 Inc W2) or other validated washer (Denley Wellwash [Soak:1, Pause:6, Washes:4, Vol:8]) and wash cycle. Blot/tap plate to remove any residual wash solution in wells.
4) Prepare oral fluid specimens by vortexing briefly and then microfuging (3,500 rpm for 10 mins) and add 100µl samples (in duplicate for first 1,000 specimens) to assigned wells on the plate.
5) Also run a dilution series (1/50-1/25600) of NoV positive serum using columns 1 & 2 of plate. Also include assay buffer control (2 wells).
6) Cover plate with a plate sealer and incubate at 37°C for 2hrs in a moist box.
7) Wash plate (as described above in step 3) and add 100µl 1:500 europium-conjugated anti-human IgG antibody (e.g. as stock concentration is 50µg/ml, add 20µl antibody to 10ml assay buffer).
8) Incubate plate(s) at 37°C for 2hr in a moist box.
9) Wash plate (as described above in step 3) and add 150µl neat enhancement solution.
10) Incubate plate whilst shaking on an orbital shaker for 10mins at room temperature in a dark environment.
11) Read plate using either/both fluorimeters using the standard test programs.

*IQC should be the standard serum giving counts of 10,000-15,000.

Reagents	**Location**
Plates	3C30
rGV antigen: Add 80ul of 1.6mg/ml to 60ml coating buffer	3C22 (4-8^{o}C)
Coating buffer	Cold room
Standard curve	-40^{o}C 3C22
Assay buffer: in use	4-8^{o}C 3C22
Assay buffer :stock	TRFIA room (EM Suite)
Wash concentrate: in use	4-8^{o}C 3C22
Wash concentrate: stock	TRFIA room (EM Suite)
Enhancement solut: in use	4-8^{o}C 3C22
Enhancement solut: stock	TRFIA room (EM Suite)
Anti-Hu IgG EU conjugate	4-8^{o}C 3C22