JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH

How to cite this article :
Halder A, Fauzdar A. POTENTIAL USE OF BLOOD, BUCCAL AND URINE CELLS FOR RAPID NONINVASIVE DIAGNOSIS OF SUSPECTED ANEUPLOIDY USING FLUORESCENCE IN SITU HYBRIDIZATION (FISH). Journal of Clinical and Diagnostic Research [serial online] 2007 April [cited: 2007 April 2]; 2:32-38.
Available from
http://www.jcdr.net/back_issues.asp?issn=0973-709x&year=2007&month=April&volume=1&issue=2&page=32-38&id=23

ORIGINAL ARTICLE / RESEARCH

Potential Use Of Blood, Buccal And Urine Cells For Rapid Noninvasive Diagnosis Of Suspected Aneuploidy Using Fluorescence In Situ Hybridization (FISH)

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ABSTRACT

Objective: The objective of the study was to determine whether noninvasive and readily available cells could be used for rapid diagnosis of specific chromosomal abnormality to facilitate management of patients in acute/specialized situation.

Methods: In the present study we analyzed blood, buccal & urine cells from 3 patients with pure trisomy 21 with locus specific FISH probes for chromosome 21. Three normal subjects were included for comparison. The clinical cases were confirmed with conventional cytogenetic studies of peripheral lymphocytes before inclusion in the study.

Result: Average frequencies for 1, 2 and 3 hybridization signals were 2.2%, 6% and 91% for blood cells, 2.5%, 7% and 89.8% for buccal cells and 2%, 9.3% and 87.4% for urine cells, respectively in the cases. False trisomic cells were less than 1% in all type of cells in controls. False monosomic cells were 3.6%, 4.5% & 9.8% for blood, buccal & urine cells in controls which was quite similar to alphoid FISH probes (chromosome 1 & 18).

Conclusion: This study suggests DNA locus specific FISH probes can be used in blood/buccal/urine cells for rapid noninvasive diagnosis, but because of high rates of false monosomy, it may not be accurate to diagnose low (<10%) level of mosaicism. The test is suitable for medically urgent situations for management guidance.

Key words: blood cells, buccal cells, urine cells, rapid noninvasive interphase FISH

Key message: Interphase FISH on blood, buccal and urine cells is a rapid, effective and non-invasive method for the diagnosis of chromosome aneuploidy.

Introduction

Chromosomal abnormality is the major cause of reproductive wastage, congenital malformation, mental retardation and cancer. Cytogenetics is indicated to diagnose a known suspected chromosomal syndrome, unexplained psychomotor retardation, sexual development anomaly, infertility, dysmorphism, cancer, recurrent pregnancy loss and pregnancy at risk for aneuploidy. Cytogenetic analysis by conventional chromosomal banding techniques, although an important standard method, requires cell culture, skilled personnel and is labor as well as time

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intensive. Interphase FISH has expanded the possibilities for precise and rapid diagnosis in clinical genetics including cancer [1],[2]. This approach was tested on peripheral blood cells [3],[4], buccal smears [3]-[8] urine smears [9],[10] and cervical smears [11],[12]. The rationale for the use of such smears is that they are noninvasive, rapid and direct i.e., tissue culture is eliminated. They are important in situations like acutely sick newborns with a suspected aneuploidy or suspected microdeletion syndrome requiring intensive therapy and newborns with ambiguous genitalia requiring quick sex assignment for management as well as social reason. Chromosome analysis by conventional method is impossible in non-dividing terminally differentiated cells viz., buccal cells, urine cells, etc or may be difficult to perform in severely ill patients in particular acutely sick newborn where venous blood sampling is difficult. With FISH technique it is possible to identify the number of specific chromosomes in interphase cells in almost all type of tissue including buccal cells, urine cells or cells from heel prick blood. Furthermore, buccal cells, urine cells or heel prick blood cells represent ectodermal (buccal cells), endodermal (urine cells) and mesodermal (blood cells) origin. In addition, a large number of interphase cells (viz. few hundreds) can be evaluated in short period of time with interphase FISH. Hence this approach is most appropriate method for mosaicism study as well. However, use of direct smears is frequently associated with poor hybridization due to difficulties in accessibility of probes and associated debris. The present study is designed to overcome the above difficulties and at the same time to maintain all the benefits of smears with most difficult locus specific FISH probes. The utility and potential are explored and discussed.

Materials and Methods

Patients

Three children between 9-12 years with features of trisomy 21 and cytogenetically proven pure trisomy 21 cases were selected from the Medical Genetics department of SGPGIMS, Lucknow. Peripheral venous blood samples from three matched cytogenetically normal males were included for comparison. Cytogenetics was performed with routine GTG banding and there were no abnormalities.

Sample Processing

Finger Prick Blood:

About 100ul blood was collected with a micropipette into a 1.5ml micro centrifuge tube containing 400ul PBS (phosphate buffered saline, pH7.4, Sigma), mixed well and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and about 500ul PBS was added, mixed and centrifuged. This washing procedure was repeated three times. About 400ul hypotonic solution was added to the pellet, mixed well and incubated for 20 minutes in 50mMol KCl before adding 100ul fresh fixative (3:1 methanol and acetic acid) and then centrifuged. The supernatant was discarded and 1000ul fixative was added into the cell pellet, mixed and centrifuged. This was repeated three times before dissolving into 50-100ul fresh fixative depending on the cell pellet size. Approximately 15-20ul of cell suspension was used for slide preparation.

Mouth Wash:

Patients were asked to rinse the oral cavity with tap water 2-3 times to minimize contamination by food particles and microorganisms before providing a mouth wash sample into a 50ml plastic tube. The tube was centrifuged at 5000 rpm for 5 minutes. The cell pellet was washed 3 times in PBS and transferred to a 1.5ml microcentrifuge tube. About 1000ul hypotonic solution was added to the pellet, mixed well and incubated for 20 minutes in 50mMol KCl before adding 100ul fresh fixative (3:1 methanol and acetic acid) and then centrifuged. The supernatant was discarded and 1000ul fixative was added into the cell pellet, mixed and centrifuged. This was repeated three times before dissolving into 100-300ul fresh fixative depending on the cell pellet size. Approximately 15-20ul of cell suspension was used for slide preparation.

Urine Sample:

A mid stream urine sample was collected into a 50ml plastic centrifuge tube. Urine cells were washed three times in PBS, hypotonic treatment was given for 20 minutes and the pellet fixed using 3 washes in fixative as with mouth wash. Finally, cells were re-suspended in 50-100ul fresh fixative depending upon the cell pellet size.

Slide Preparation

Approximately 10ul cell suspension was applied onto each clean un-coated microscopic glass slide. The slide was then treated with 70% acetic acid for 1 minute (for blood & urine cells) or 2 minutes (buccal cells) and dehydrated in alcohol series (70%, 90% & 100% ethanol) for three minutes in

each. The fixed cells on slides were treated for 20 minutes at 37ºC in pepsin solution (10 ug/ml, pH 1.5-2.0), washed in water, fixed in 1% paraformaldehyde at 4ºC for ten minutes and finally dehydrated as before.

FISH Probes

Chromosome 21 locus specific probe was commercially purchased from Vysis Inc. (France) whereas chromosome 1 & 18 alphoid probes were obtained from Uniba Biologia, Italy.

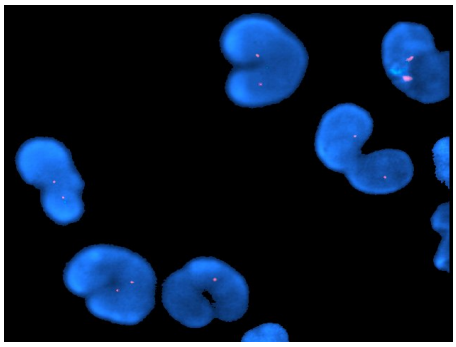
Probe preparation & FISH

Probe preparation, hybridization, post hybridization washing and visualization was as per the Vysis protocol (www.vysis.com). Probes and cell DNA were denatured together for 3 minutes (for blood & urine cells) or 5 minutes (for buccal cells) at 75ºC in an oven and incubated at 37ºC in a moist chamber for 12-18 hours (overnight). During the study, lymphocyte metaphase spreads were used to check hybridization specificity & efficiency. Post hybridization washing was done as per the Vysis protocol using NP40. The slides were counterstained & mounted using antifade containing DAPI before screening under a Nikon Optiphot/Olympus BX51 fluorescent microscope. Images were captured using a digital imaging system (Applied Imaging, UK). Non-hybridized cells were excluded from the study.

Results

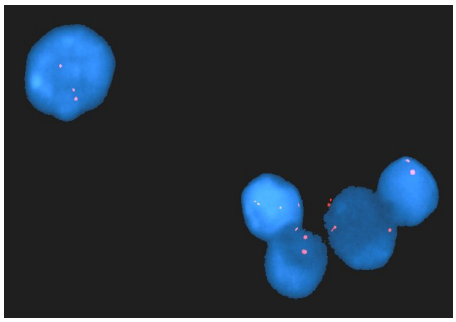
The hybridization efficiency based on metaphase evaluation of probes was 100%, and no cross-hybridization occurred. The interphase hybridization efficiency of probes on all types of cells was over 90%. Approximately a few hundred nuclei each from blood (857), buccal (1002) & urine cells (397) were scored from three trisomy 21 cases with the chromosome 21 locus specific probe. The expected number of signals was obtained in 91%, 89.8% and 87.4% nuclei with blood, buccal and urine cells respectively [Table/Fig 5], [Table/Fig 6], [Table/Fig 7]. This result was comparable with chromosome 1 & 18 alphoid probes [Table/Fig 8]. Figures show signals obtained with blood [Table /Fig 1], [Table /Fig 2], buccal [Table/Fig 3] and urine [Table /Fig 4] samples with the chromosome 21 locus specific probe.

Table/Fig 1



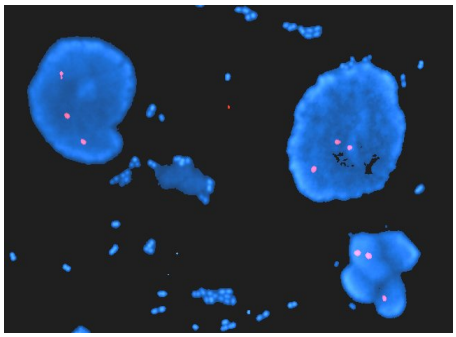
Interphase FISH using locus-specific chromosome 21 probe on finger prick sample. Signals mostly on polymorphs/neutrophils from normal sample.

Table/Fig 2



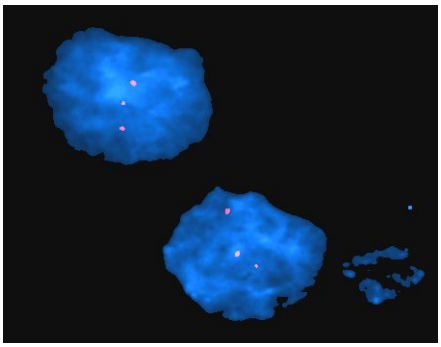
Interphase FISH using locus-specific chromosome 21 probe on finger prick sample. Signals mostly on lymphocytes from trisomy 21 case.

Table/Fig 3



Interphase FISH using locus-specific chromosome 21 probe in a trisomy 21 case on buccal cells (note debris & microorganisms besides squamous cell nuclei).

Table/Fig 4



Interphase FISH using locus-specific chromosome 21 probe in a trisomy 21 case on urine cells.

Discussion

The intent of this study was to determine whether blood, buccal & urine cells could be used for specific diagnosis for facilitating management of patients. This study indicates that interphase FISH with locus specific probes can be used successfully on these preparations. The technique is simple, noninvasive and quick providing results in few hours (alphoid probe) to 24 hours. The cells are easily available and can be collected without trauma by noninvasive/minimally invasive techniques. As the procedure was rapid, it can be of extreme help in situations like ambiguous genitalia where assignment of sex is required in a few hours for social reasons as well as for appropriate management [4],[6]. It has similar value in an acutely sick baby with malformations suggestive of trisomy 21/18/13/etc [4] or microdeletions (DiGeorge, Velo Cardio Facial, etc) [7] requiring urgent operation/intensive care.

Mosaicism is a common condition among patients with a chromosome abnormality, in particular, aneuploidy. Our study with the above cells indicates that FISH with chromosome 21/1/18 probes is sufficient to detect monosomic cells constituting >10% of the total cell population or trisomic cells constituting >5% of total cell population. However, low level of mosaicism (<10%) cannot be diagnosed confidently particularly with urine cells. Buccal cells are derived from ectoderm. Urine cells are mostly derived from endoderm whereas blood cells are from mesoderm. Interphase FISH using these cells is appropriate to detect tissue specific mosaicism because the extent of mosaicism is known to vary significantly among different tissues. Pallister-Killian syndrome (PKS), a rare disorder, is characterized by tissue-limited or tissue-specific mosaicism. The characteristic chromosome abnormality associated with PKS is i(12p), which is seen predominantly in skin fibroblast cultures. Diagnosis of i(12p) on buccal smears was shown to be an easy and feasible method [13].

Change in chromosome number (aneuploidy) has been consistently linked with many genetic disorders including cancer. Since 90% of cancers arise in epithelial tissue, which is difficult to culture, techniques that measure aneuploidy in these tissues i.e., FISH would be very useful. Here this study suggests FISH can be used to detect aneuploidy in exfoliated epithelial cells collected from the mouth (buccal) and bladder (urine). Since chromosomal aberrations are involved even in pre-cancerous conditions, FISH on buccal cells of betel quid chewers can be used for screening [14]. Similarly, this can be extended for monitoring exposure to genotoxic agents including effect of hyperstimulating drugs on ovarian follicular cells. Recently, Madon et al. [15] has reported feasibility of the use of cumulus cells (granulosa cells) obtained through ovum pick-up for aneuploidy study by interphase FISH.

There are some drawbacks in interphase FISH. We did not find the expected number of signals in all types of cells similar to Jenkins et al [16]. Many factors can be attributed to this, including uptake of probe through the cell membrane particularly with buccal cells (squamous cells). Harris et al [5] used buccal smears from trisomy 21 cases by interphase FISH. They reported a probe efficiency of 71%. In this study the probe efficiency was above 90% for the locus specific probe which is more difficult to hybridize. This difference may be due to use of smears where release of nuclei and hypotonic effect are difficult. Protein digestion with pepsin in our study was found to be efficient in removing the keratinized cell membrane and optimizing probe penetration. A 30 min digestion with 300 micrograms/ml of pepsin in 0.01 M HCl optimized probe penetration in buccal cells. Furthermore 2-3 mouthwashes prior to collection removed most of the superficial and dead cells with degraded DNA thus resulting in a low monosomy rate. We observed consistently low yield of cells as well as high monosomy in urinary cells with both locus specific and alphoid probes. This could be due to presence of few good cells.

In conclusion, interphase FISH on blood, buccal and urine cell is a rapid, effective and non-invasive method for the diagnosis of chromosome aberrations. This test is very specific and reliable when used with a strong clinical suspicion.

Table/Fig 5. Result of interphase FISH with locus-specific chromosome 21 probe on blood cells obtained from finger prick. Nuclei without signal were excluded from analysis. Overall hybridization efficiency was over 90%. Disparity between total number and combined mono/di/tri signals is due to presence of some nuclei with 4-6 signals

Parameters	Total nuclei	One Signal (%)	Two Signals (%)	Three Signals (%)	Remark
Trisomy Cases					
Case 1	296	8	22	265	
Case 2	215	5	17	192	
Case 3	346	6	13	324	
Total	857	19 (2.2%)	52 (6%)	781 (91%)	
Remark					Trisomy 21
Control Cases					
Case 1	248	7	237	2	
Case 2	125	3	120	1	
Case 3	153	9	141	1	
Total	526	19 (3.6%)	498 (94.7%)	4 (0.76%)	
Remark					monosomic cells <4%

Table/Fig 6. Result of interphase FISH with locus-specific chromosome 21 probe on buccal cells obtained from mouthwash. Nuclei without signal were excluded from analysis. Overall hybridization efficiency was over 90%. Disparity between total number and combined mono/di/tri signals is due to presence of some nuclei with 4-6 signals

Parameters	Total nuclei	One Signal (%)	Two Signals (%)	Three Signals (%)	Remark
Trisomy Cases					
Case 1	414	10	39	362	
Case 2	369	13	26	327	
Case 3	219	02	05	211	
Total	1002	25 (2.5%)	70 (7%)	900 (89.8%)	
Remark					Trisomy 21
Control Cases					
Case 1	113	5	105	2	
Case 2	223	9	211	1	
Case 3	110	6	102	1	
Total	446	20 (4.5%)	418 (93.7%)	4 (0.89%)	
Remark					monosomic cells <5%

Table/Fig 7. Result of interphase FISH with locus-specific chromosome 21 probe on urinary tract cells obtained from urine samples Nuclei without signal were excluded from analysis. Overall hybridization efficiency was over 90%. Disparity between total number and combined mono/di/tri signals is due to presence of some nuclei with 4-6 signals

Parameters	Total nuclei	One Signal (%)	Two Signals (%)	Three Signals (%)	Remark
Trisomy Cases					
Case 1	146	4	19	121	
Case 2	127	2	08	115	
Case 3	124	2	10	111	
Total	397	8 (2%)	37 (9.3%)	347 (87.4%)	
Remark					Trisomy 21
Control Cases					
Case 1	102	14	085	2	
Case 2	084	06	076	0	
Case 3	129	11	116	1	
Total	315	31 (9.8%)	277 (87.9%)	3 (0.95%)	
Remark					monosomic cells <10%

Table/Fig 8. Result of interphase FISH with alphoid probes (Chromosome 1: red & 18: green dual color) on blood, buccal and urine cells in normal control. Nuclei without signals were excluded from counting. Overall hybridization efficiency was noted in over 90% nuclei (blood 98.6%, buccal 98.4% & urine 90.2%)

Parameters	Total nuclei	Monosomy 1/18/both (%)	Two Signals both 1 & 18 (%)	Three Signals 1/18/both (%)	Remark
Blood Cells	289	04	0285	0	1.4% monosomic cells
Buccal Cells	1077	14	1060	3	1.3% monosomic cells
Urine Cells	92	07	0085	0	7.6% monosomic cells

In cases where the FISH result is consistent with the phenotype, no further studies are required. However, a follow-up with complete chromosome analysis should be recommended for comparison and additional information whenever possible due to the limitations inherent in interphase cytogenetics and DNA probes.

Acknowledgement

The authors thank Professor Mariano Rocchi (University of Bari, Italy) for resources on

molecular cytogenetics (plasmid clones for chromosome 1 & 18). We also thank Dr J Pahi (ex senior resident) and Mr. Ramsaran (technical officer, cytogenetics) of the Department of Medical Genetics, for providing various assistances during the study.

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