

# RAPID EARLY PREGNANCY SEXING BY CHORIONIC VILLUS BIOPSY AND QUINACRINE STAINING

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## SUMMARY

*Rapid and reliable sexing of first-trimester pregnancies is possible by quinacrine dihydrochloride staining of chorionic villi obtained by ultrasound guided chorionic villus biopsy.*

*Chorionic villus biopsy was performed in 20 cases scheduled for termination of pregnancy for social reasons, the average gestational age was 8.4 weeks with a range of seven to 11 weeks.*

*The average time taken for the procedure starting from cleaning the vagina to identification of the villi under the dissecting microscope was 12.7 minutes with a range of five to 30 minutes.*

*Chorionic villi material was stained with a 0.25% aqueous solution of quinacrine dihydrochloride for five minutes and inspected for 'Y' bodies after mounting in distilled water.*

*For ten chromosomally-confirmed XY cases, the mean 'Y' count was  $32.7\% \pm 12.2\%$ .*

*For ten chromosomally confirmed XX cases, the 'Y' body count was  $3.5\% \pm 2.7\%$ .*

*XY cases had significantly higher 'Y' body counts than XX cases.*

## INTRODUCTION

Curiosity about the sex of the foetus *in utero* has been present ever since history began.

Sexing during pregnancy has become clinically relevant because of the understanding of sex-linked diseases and the ability to perform selective termination of pregnancy in at-risk cases.

Sexing during the second trimester is well-established by means of amniocentesis and karyotyping of amniotic fluid cells.

Termination of second trimester pregnancy is unpleasant and carries higher risk than first-trimester termination.

With the recent introduction of first-trimester chorionic villus biopsy, foetal sexing is now made possible in early pregnancy.

This paper proposes to demonstrate a rapid method of early foetal sexing by combining ultrasound guided chorionic villus biopsy with the cytogenetic sexing method of quinacrine dihydrochloride staining.

## METHOD

Twenty consecutive patients who had viable singleton pregnancies between seven and 11 weeks gestation (mean  $8.4 \pm 2.1$  weeks) as assessed by ultrasound were recruited into this study.

All had requested for termination of pregnancy for social reasons. The patients' ages ranged from 18 to 38 years, with a mean of 24 years; 14 were nulliparae, 17 had anteverted uteri and 12 had anterior placentae.

Informed consent was obtained from the patients. Chorionic villus biopsy was performed immediately prior to standard first-trimester termination of pregnancy.

Patients were given 10mg intravenous diazepam (Valium, Roche) prior to the procedure. A real-time ultrasound scanner (Hitachi EUB 40), fitted with a 40MHZ convex-array transducer was used to assess the size and viability of the foetus, the position of the uterus and the position of the placenta.

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The biopsy cannula (Trophocan, Portex, Kent, England) was then bent to fit the angle formed by the cervical canal and uterus.

After noting the starting time, the vulva and vagina were cleaned with hibitane solution and the cervix exposed by a Sim's speculum held by an assistant. The anterior lip of the cervix was established with a vulsellum held by the assistant.

The ultrasound transducer was then held by the operator's left hand while the biopsy cannula was held by the right hand. With the image of the cannula tip being observed by ultrasound, the cannula was guided through the cervical canal and to the centre of the placental site.

The operator then removed the trocar and attached a 20cc disposable plastic syringe (Terumo, Japan) to the cannula. The plunger was then withdrawn by 5 to 10 ml creating a vacuum suction at the cannula tip. The cannula was then withdrawn under suction to avulse the chorionic villi. The amount of material removed was estimated by the length of the cannula it occupied. This was standardized by prior testing: (1cm = 10 $\mu$ g).

The presence of foetal heart movements was confirmed by ultrasound after the procedure.

The end point in timing the procedure was taken as the time when chorionic villi were confirmed by inspecting under a dissecting microscope (Zeiss Stereo-microscope SV8).

Chorionic villi were identified as frond-like material<sup>1</sup> when the suspension of villi in Hartman's Ringer Lactate solution (Otsuka, Thailand), was inspected under 8 to 25X magnification with a dissecting microscope (Fig. 1).

Blood clot and amorphous tissue (which could be maternal residue) were removed from the specimen with microsurgery forceps. Half of the chorionic villi of each case was sent in culture medium (modified Tyrode's solution, HT6)<sup>2</sup> for chromosome culture and karyotyping by the Cytogenetics Laboratory, Department of Paediatrics, National University of Singapore.

The other half was rinsed with Hartman's solution and placed on three replicate slides. After termination of pregnancy, chorionic villi

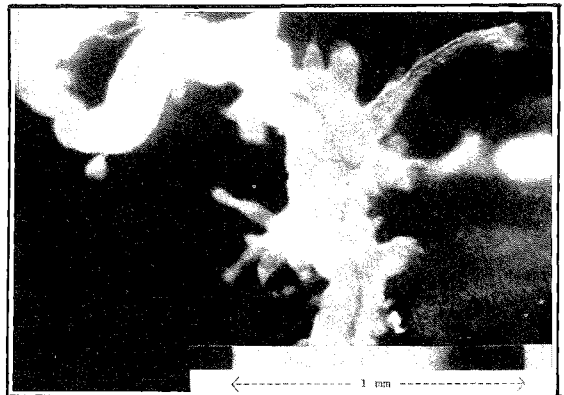


Fig. 1 Chorionic villi (64X magnification).

material was plucked from the placental mass, rinsed with Hartman's solution and placed on three replicate slides.

Each of the specimens was then squashed with a coverslip and dried on a 37<sup>o</sup> dry block. They were then fixed by dropping a 3:1 mixture of the absolute methanol and glacial acetic acid over the inclined slides. The slides were then air dried and stored. After every three consecutive cases, the replicate slides were randomly numbered according to a table of random numbers,<sup>3</sup> stained and inspected for 'Y' bodies.

Staining was performed by immersing the slides for five minutes in freshly-prepared 0.25% solution of quinacrine dihydrochloride (Sigma, St Louis Mo, USA) in distilled water at room temperature. The nuclei of the cytotrophoblast take up the quinacrine dihydrochloride stain very well and have a tendency of being overstained with the 0.5% solution. A 0.25% solution provides a better stain intensity for visualising the 'Y' body.

The slides were then rinsed in distilled water and coverslips placed over the wet slides. The cytotrophoblast nuclei of the chorionic villi were identified by their characteristic oval shape on the periphery of the villi fronds.<sup>4</sup>

The presence of the small, brightly fluorescent, 'Y' body within each nucleus was looked for with a Zeiss Standard microscope which was fitted with an HBO 50 mercury light source. The filter assembly consisted of a 436/8nm narrow band-pass excitor filter and a 470/nm barrier filter providing epifluorescence. The objective lens used was a 100x in oil, plan-apochromatic lens with an

iris-diaphragm which was stopped down until an optimal image of the cytotrophoblast nuclei was obtained.

One hundred nuclei were inspected from each slide and the number of nuclei containing a centrally-located, small, brightly fluorescent 'Y' body counted (Figs. 2, 3). The results of karyotyping were then obtained. The 'Y' body counts of the 20 chorionic-villus biopsy cases are tabulated in Table I according to chromosomal sex. 'Y' body counts from XY cases were compared with the XX cases using Wilcoxon's Rank Sum Test and Student's Unpaired 'T' Test, for significance of difference.

In order to determine whether the chorionic villus material obtained by biopsy corresponded to the placental material obtained on termination of the same pregnancy, 'Y' body counts of the two sets of data were compared in Table II.

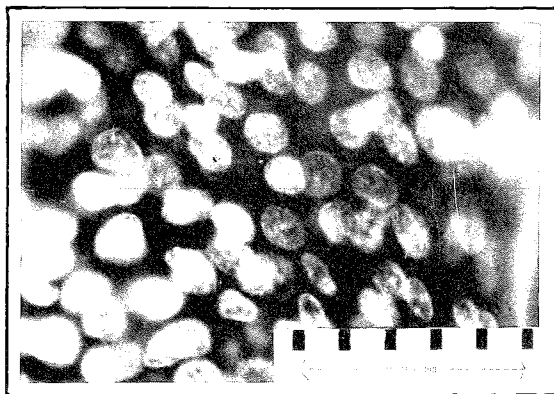


Fig. 2 Y-Body positive trophoblast nuclei: 1000X magnification.

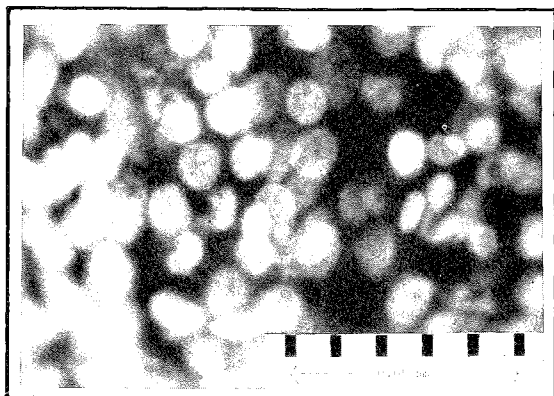


Fig. 3 Y-Body negative trophoblast nuclei: 1000X magnification.

TABLE I  
'Y' BODY COUNTS OF NUCLEI FROM  
BIOPSIED VILLI

XY Cases				XX Cases			
% Y Body count :				% Y Body count			
1	2	3	Mean	1	2	3	Mean
25	28	36	29.6	3	3	5	3.6
35	24	25	28.0	3	2	2	2.3
42	52	44	46.0	3	4	3	3.3
21	38	21	26.6	4	3	6	4.3
39	26	24	29.6	3	2	6	3.6
28	35	32	31.6	1	3	2	2.0
34	20	28	27.3	3	5	2	3.3
42	43	36	40.3	4	9	5	6.0
30	42	29	33.6	2	1	1	1.3
31	33	38	34.0	5	4	7	5.3

Mean : 32.7% ± 12.2%                      Mean : 3.5% ± 2.7%

Wilcoxon's Signed Rank Test and Student's Paired 'T' Test were used to test for any significant difference between the paired data.

## RESULTS

The mean duration of the procedure of chorionic villus biopsy was 12.7 minutes with a range of five to 30 minutes. Of the 20 cases studied, difficulty in insertion of the cannula was encountered in two nulliparous patients and one parous patient.

The mean number of insertions per patient was 2.4 times with a range of 1 - 5 times. The mean number of aspirations was two times with a range of 1 - 4 times.

Bleeding during and immediately after the procedure was absent in six patients, minimal in 12 cases, moderate in two. The mean amount of chorionic villi obtained was estimated to be 26  $\mu$ g with a range of 10 to 40  $\mu$ g.

Table I shows the 'Y' body counts of 20 chorionic villus biopsy cases tabulated according to chromosomal sex. 'Y' body counts of XY cases

TABLE II  
 'Y' BODY COUNTS FROM NUCLEI OF BIOPSED  
 CHORIONIC VILLI AND FROM PLACENTAL  
 MATERIAL OBTAINED BY TERMINATION  
 OF PREGNANCY

Biopsy material				Termination material			
% 'Y' Body count				% 'Y' Body count			
1	2	3	Mean	1	2	3	Mean
3	3	5	3.6	2	4	1	2.3
3	2	2	2.3	2	1	4	2.3
3	4	3	3.3	4	4	5	4.3
25	28	36	29.6	39	22	27	29.3
4	3	6	4.3	4	5	4	4.3
35	24	25	28.0	27	34	22	27.6
42	52	44	46.0	59	53	37	49.6
3	2	6	3.6	5	4	3	4.0
1	3	2	2.0	6	3	1	3.3
3	5	2	3.3	5	2	4	3.6
21	38	21	26.6	31	20	29	26.6
39	26	24	29.6	31	32	26	29.6
4	9	5	6.0	3	6	4	4.3
2	1	1	1.3	0	2	1	1.0
28	35	32	31.6	38	34	30	34.0
34	20	28	27.5	29	34	21	28.0
5	4	7	5.3	2	8	1	3.6
42	43	36	40.3	45	34	42	40.3
30	42	29	33.6	36	29	32	32.3
31	33	38	34.0	36	35	33	34.6

were significantly higher than XX cases ( $P < 0.001$ , Wilcoxon's Rank Sum Test, where  $n_1 = n_2 = 10$  and rank sum  $XY:XX = 55:210$ )<sup>5</sup> ( $P \leq 0.001$ , Student's 'T' Test, where  $t = 6.6$ ). Table I also shows that there is no case in which the highest 'Y' body count of the ten XX cases overlapped the lowest 'Y' body count of the XY cases.

Table II shows that the 'Y' body counts of chorionic villus biopsy material in 20 cases are not significantly different from the counts from the placental material obtained from the same pregnancy after termination. ( $P > 0.5$ , Wilcoxon's Signed Rank Test, where  $n = 13$  and sum of negative-signed ranks = 64 and sum of positive-signed ranks = 27.)<sup>5</sup> ( $P \geq 0.01$ , Student's 'T' Test, where  $t = 0.213$ .)

## DISCUSSION

The technology of determining the sex of interphase nuclei began with the demonstration of the chromatin body in female cells by Barr and Bertram in 1949.<sup>6</sup> The staining method was improved by Klinger in 1957 with Thionin.<sup>7</sup> Another milestone is chromosome culture and karyotyping in peripheral blood leukocytes by Moorhead *et. al.*, in 1960.<sup>8</sup>

In 1970, Caspersson *et. al.*, demonstrated that quinacrine dihydrochloride and quinacrine mustard selectively stained the 'Y' chromosome.<sup>9</sup> This pioneering work has been confirmed by other workers.<sup>10-15</sup> These techniques have been applied to the task of determining the sex of the foetus. The problem is to be able to gain access to the products of conception without damage to the foetus.

Attempts have been made to identify cells of male origin in maternal blood in order to identify the presence of a male foetus *in-utero*.

One method is to stain for the 'Y' chromosome in lymphocytes obtained from maternal blood.<sup>16</sup> Another is to isolate male cells of foetal origin by fluorescence activated cell sorting.<sup>17</sup> However, both these methods are not accurate enough for clinical application.

Another approach is to sample cells from the endocervix of pregnant woman in the hope of identifying foetal cells which have desquamated from the conceptus. Although this method is claimed to be effective,<sup>13,18</sup> it is still not considered reliable enough for clinical application.<sup>19-22</sup>

Sexing of the foetus in the second trimester is well established.<sup>23-26</sup> Amniotic fluid is obtained by amniocentesis and the cells obtained by centrifugation are stained with Aceto-orcein for the 'X' chromatin,<sup>27</sup> or by quinacrine salts for the 'Y' body.<sup>27-29</sup> The amniotic fluid cells can also be cultured and karyotyped.<sup>30,31</sup> Direct visualisation of the mid-trimester foetus has been achieved by amnioscopy.<sup>32</sup> However, this can only be achieved in expert hands to avoid a high miscarriage rate. More recently, ultrasound guided foetal cord blood sampling has been achieved.<sup>33</sup>

The main disadvantage of foetal sexing in mid-trimester is that when a foetus at risk is identified,

the termination procedure is associated with a relatively high maternal risk when compared to a termination done in the first trimester. First trimester sexing has been attempted in order to avoid the risks associated with termination of mid-trimester pregnancies.

Earlier studies using transcervical endoscopic methods were not encouraging probably due to problems in the technique of cell culture<sup>34</sup> or of the technique of biopsy of the chorionic tissue.<sup>35</sup> One study showed a 93% accuracy of sexing using Papanicolaou staining of chorionic cells obtained by blind aspiration.<sup>36</sup>

Blind transcervical aspiration of chorionic tissue was not uniformly successful and there was, often, maternal tissue contamination.<sup>37,38</sup> Moreover, this method may cause damage to the amniotic sac and contents.<sup>38,39</sup> Accuracy of sexing has been improved by various methods.<sup>40-44</sup>

Han *et. al.*, first described the application of quinacrine dihydrochloride to stain for the 'Y' body in chorionic villi.<sup>45</sup> This has been confirmed by Kazy *et. al.*<sup>46</sup> In 1981, Niazi *et. al.*, demonstrated good cultures obtained from trypsin processed chorionic villi material.<sup>47</sup> Simoni *et. al.*, in 1983 described a rapid method of chromosome culture and karyotyping,<sup>48</sup> but the method is difficult to master. Further accuracy was achieved by use of DNA probes for the 'Y' chromosome.<sup>49</sup>

The advantage of 'Y' body detection as compared to karyotyping is the relative simplicity and rapidity of the method. The method of staining described in this paper can provide results within half an hour of obtaining the chorionic villi specimen. The time taken for ultrasound guided chorionic villus biopsy depends on the operator's experience and can be reduced to within ten minutes from the start of the procedure.

There are several pitfalls in the first trimester foetal sexing. Maternal tissue contamination is a problem but this can be reduced by proper identification of the characteristic villi fronds when suspended in water<sup>1</sup> and removing residual fragments using an inverted phase-contrast microscope.<sup>48,50</sup>

Mosaicism in the cell culture may confuse results,<sup>51,52</sup> but this problem can be reduced by direct chromosome analysis<sup>48</sup> or direct staining for the 'Y' body. In cases of familial deletion of the long arm of the 'Y' chromosome, 'Y' body staining may be negative even if it is a male foetus.<sup>53</sup>

Clinical application of first trimester foetal sexing is now established for Duchenne muscular dystrophy,<sup>54-56</sup> for haemophilia<sup>56</sup> and is likely to become established for other sex-linked diseases.

More accurate diagnosis of these diseases *in utero* is possible by mid-trimester sampling of foetal blood by ultrasound guided placental punctures,<sup>57</sup> by foetoscopy<sup>32</sup> or by ultrasound, guided foetal cord blood sampling.<sup>33</sup> These relatively high-risk mid-trimester procedures can be avoided in cases detected to be female by first trimester sexing using ultrasound guided chorionic villus biopsy and quinacrine staining.

## CONCLUSION

Rapid and reliable sexing of first trimester pregnancies is possible by quinacrine dihydrochloride staining of chorionic villi material obtained by ultrasound guided chorionic villus biopsy.

## ACKNOWLEDGEMENTS

The author would like to thank Professor H. B. Wong and Mr T. S. Chua of the Cytogenetics Laboratory, Department of Paediatrics, National University of Singapore, for performing the karyotyping of the chorionic villi and Mdm T. K. Cheong for typing the manuscript.

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