Rapid aneuploidy screening with fluorescence in-situ hybridisation: is it a sufficiently robust stand-alone test for prenatal diagnosis?

Objectives
To assess the clinical utility of fluorescence in-situ hybridisation with chromosomes 13, 18, 21, X and Y as a stand-alone test in detecting chromosomal abnormalities, and the types of chromosomal abnormalities missed.

Design
Retrospective analysis.

Setting
A restructured Government hospital in Singapore and an academic hospital in the United States.

Participants
Cytogenetic data of prenatal specimens and results of fluorescence in-situ hybridisation of 5883 patients performed between January 2000 and August 2007 were reviewed.

Results
Fluorescence in-situ hybridisation detected 558 (9.5%) patients with chromosomal abnormalities. Abnormal ultrasounds (70%) and maternal serum screens (21%) were the most indicative of chromosomal abnormalities. When comparing fluorescence in-situ hybridisation data with karyotype results for the five chromosomes of interest, the sensitivity and specificity were 99.3% and 99.9%, respectively. When comparing fluorescence in-situ hybridisation data with karyotype results for all chromosomes, the sensitivity decreased to 86.8%, whereas the specificity remained at 99.9%. Of 643 cases with karyotype abnormalities, 85 were fluorescence in-situ hybridisation–negative (false negative rate, 13.2%), which included structural rearrangements, mosaicism, and other trisomies. Despite abnormal ultrasound indications, fluorescence in-situ hybridisation missed 32 cases which included structural rearrangements, mosaicism, and other trisomies.

Conclusion
This study does not support fluorescence in-situ hybridisation as a stand-alone test. Institutions supporting fluorescence in-situ hybridisation as a stand-alone test must seriously consider the risks of a missed diagnosis.

Introduction
Cytogenetic analysis of fetal cells has been the standard test in prenatal diagnosis. This method involves the acquisition of metaphase chromosomes through a period of cell culture that may take anywhere between 7 and 14 days. Despite increasingly shorter prenatal result turn-around times due to the wider adoption of the in-situ coverslip technique and improved culture media, patients remain anxious while waiting for the 1 to 2 weeks it takes for an amniotic fluid (AF) or chorionic villus sampling (CVS) karyotype result.

Consequently, rapid aneuploidy screen (RAS) tests such as fluorescence in-situ hybridisation (FISH) and quantitative fluorescence–polymerase chain reaction (QF-PCR) assays to detect numerical abnormalities of chromosomes 13, 18, 21, X and Y have become increasingly popular adjunct tests. Unlike karyotyping, these test results are typically available within a few hours to 2 days, thereby alleviating much of the anxiety from these patients. Since aneuploidies of chromosomes 13, 18, 21, X and Y account for 60 to 80% of the chromosomal aberrations at the time of prenatal diagnosis, the prenatal RAS FISH assay is centred on just these five chromosomes. Commercially available probes for these five chromosomes have a reported sensitivity and specificity of around 100%. These probes have been shown to be highly accurate by several groups.
目的 評估染色體 13、18、21、X和Y熒光原位雜交術作為染色體異常獨立測試的臨床意義，以及分析漏診的染色體異常類別。

設計 回顧分析。

安排 新加坡一所重建政府醫院和美國一所學術醫院。

參與者 回顧2000年1月至2007年8月期間，5883名病人的產前檢查數據和熒光原位雜交術結果。

結果 熒光原位雜交術能檢測到558名 (9.5%) 病人呈現染色體異常。異常的超聲波 (70%) 和孕婦血清檢查結果 (21%) 最有效顯示染色體異常。把上述五種染色體的熒光原位雜交術數據與染色體核型結果比較時，敏感性和特異性分別為99.3%和99.9%。把所有染色體的熒光原位雜交術數據與染色體核型結果比較時，敏感性減至86.8%，特異性則維持99.9%。在643宗染色體異常病例中，85宗的熒光原位雜交術結果呈陰性 (錯誤負判率，13.2%)，這包括基因結構重組、鑲嵌型染色體和其他三體綜合徵。儘管超聲波呈異常結果，但熒光原位雜交術也漏診32宗基因結構重組、鑲嵌型染色體和其他三體綜合徵病例。

結論 上述研究並不支持熒光原位雜交術作為獨立測試。醫療機構若考慮使用熒光原位雜交術作為獨立測試，必須慎重考慮有漏診的風險。

以熒光原位雜交術進行快速非整倍體測試：獨立測試能否提供準確的產前診斷？

Of late, calls have been made to introduce RAS as the primary test, replacing chromosome karyotyping altogether and using it only if indicated by an abnormal ultrasound (AU).13-15 In 2004, the UK National Screening Committee (UKNSC) recommended new screening programmes for Down syndrome with FISH or QF-PCR, that could be offered as a rapid stand-alone diagnostic test instead of karyotyping. The UKNSC also suggested that it should be offered as part of the UK National Health Service provision to all women undergoing invasive testing.16

These proposals to replace karyotyping with RAS have been made on the basis of purported cost-savings for patients and more efficient utilisation of limited clinical resources. Because it is expensive to perform RAS and karyotyping on all prenatal samples, various strategies involving RAS screening have been proposed.17,18 One option is to offer FISH for chromosomes 13, 18, 21, X and Y as a stand-alone test to patients without an AU finding but with a clinical indication, namely advanced maternal age (AMA) or abnormal maternal serum screening (MSS). Karyotyping following FISH would be reserved only for those with an indication based on AU.

Despite the very high sensitivity and specificity of the assays, aneuploidy detection by FISH and QF-PCR have their own serious limitations. These include their innate inability to detect unbalanced structural rearrangements, trisomies other than chromosomes 13, 18, 21, X and Y, and numerical mosaicism that can result in birth defects. For this reason, RAS is usually performed in many laboratories as an adjunct to karyotyping.

The aim of this study was to assess the clinical utility of the FISH assay with probes for chromosomes 13, 18, 21, X and Y as a stand-alone test in detecting chromosomal abnormalities, and the types and frequencies of chromosomal abnormalities missed. To evaluate these, archival reports of cases that had both karyotype and interphase FISH tests were reviewed and analysed retrospectively.

Methods

Patients

The cytogenetic data of AF and CVS and interphase FISH analyses of patients performed between January 2000 and August 2007 at the Cytogenetics Laboratory (Singapore General Hospital, Singapore) and the Human Genetics Laboratory (University of Nebraska Medical Center, Omaha, US) were retrieved and reviewed. A total of 5883 patient samples were included in this analysis. Amniocentesis was generally performed at around 16 weeks of gestation and usually 20 mL of fluid was collected. Chorionic villus sampling was performed at round 12 weeks of gestation and usually 15 mg were obtained.

Fluorescence in-situ hybridisation

Fluorescence in-situ hybridisation probes

The FISH assay employed the AneuVysion Assay Kit (Abbott Molecular, US) comprising CEP 18 (SpectrumAqua), CEP X (SpectrumGreen), CEP Y (SpectrumOrange), LSI 13 (SpectrumGreen), and LSI 21 (SpectrumOrange) probes.

Amniotic fluid

About 2 to 5 mL of uncultured AF samples were used for each assay. Amniotic fluid samples were prepared according to the protocol recommended by the manufacturer.

Chorionic villus

The villi were cleaned under a stereomicroscope to ensure no maternal decidua remained before they were processed. Approximately 5 mg of villi were processed for each FISH assay. The chorionic villi were prepared according to the protocol recommended by the manufacturer. The slides were labelled with the lab number, patient’s initials, and FISH probes to be used.
Fluorescence in-situ hybridisation protocol

Probe mixtures were added onto the target areas of the slides, coverslipped and the edges sealed with rubber cement. Co-denaturation and hybridisation were carried out in a HYBrite system (Abbott). The next day, the slides were washed and prepared for analysis. Fifty nuclei were scored for each probe.

Results

From January 2000 to August 2007, both centres collectively processed a total of 5883 cases with both karyotyping and FISH requests.

The clinical indications for prenatal studies included AMA (≥35 years) [24.2%], AU findings (33.6%), abnormal MSS (34.9%), family history of a genetic or chromosomal disorder (5.8%), parental anxiety (0.6%), and others such as in-vitro fertilisation pregnancy. In cases where there were multiple indications, priority of indication was assigned as follows: AU, MSS, and AMA. About a third of each of all indications were for AU findings and abnormal MSS (Table 1). Conventional karyotyping was carried out concurrently for all cases. A minimum of 15 colonies or 20 cells were analysed for each case. To exclude mosaicism, two or more cultures were analysed per case.

A total of 558 (9.5%) cases of chromosomal abnormalities were detected by FISH (Table 2). Trisomy 21, as expected, was the most frequent chromosomal disorder across all indications (42.1%). Some of the other abnormalities were trisomy 18 (25.3%), monosomy X (14.0%), trisomy 13 (12.4%), triploidy (3.6%), and other sex chromosome disorders (2.2%), in descending order. The majority of the abnormalities identified by FISH were due to AU (70.1%) and MSS (21.0%); AMA constituted 6.6% of the abnormal cases while family history and other indications accounted for the remainder. There were no abnormalities detected for parental anxiety.

When comparing the FISH data with the karyotype results for chromosomes 13, 18, 21, X and Y in the 5883 individuals tested, there was only one false-positive result, but there were four false-negative results, giving a sensitivity of 99.3% and a specificity of 99.9% (Table 3). The false-positive rate was 0.02% while the false-negative rate was 0.7%. The sole false-positive case was due to a balanced translocation between chromosomes 9 and 18 at bands p11.2 and p11.1, respectively. This led to a split chromosome 18 centromere signal resulting in three interphase FISH signals. The false-negative results were due to samples that were heavily bloodstained compounded by technical difficulties.

As the FISH assay is specific and designed to detect aneuploidies of only the five chromosomes, it follows that there would be abnormalities that are undetectable by FISH when the FISH performance is compared with the karyotype results for all

---

### TABLE 1. Clinical indications for prenatal studies

<table>
<thead>
<tr>
<th>Indication</th>
<th>No. (%) of samples (n=5883)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced maternal age (≥35 years)</td>
<td>1422 (24.2)</td>
</tr>
<tr>
<td>Abnormal ultrasound</td>
<td>1974 (33.6)</td>
</tr>
<tr>
<td>Abnormal maternal serum screening</td>
<td>2054 (34.9)</td>
</tr>
<tr>
<td>Family history of genetic/chromosomal disorder</td>
<td>340 (5.8)</td>
</tr>
<tr>
<td>Parental anxiety</td>
<td>35 (0.6)</td>
</tr>
<tr>
<td>Others</td>
<td>58 (1.0)</td>
</tr>
</tbody>
</table>

### TABLE 2. Detection of abnormal cases by fluorescence in-situ hybridisation according to various indications

<table>
<thead>
<tr>
<th>Indication</th>
<th>Trisomy 13</th>
<th>Trisomy 18</th>
<th>Trisomy 21</th>
<th>Monosomy X</th>
<th>XXY</th>
<th>XXX</th>
<th>XYY</th>
<th>Triploidy</th>
<th>Mosaicism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced maternal age (≥35 years)</td>
<td>0</td>
<td>8</td>
<td>26</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal ultrasound</td>
<td>62</td>
<td>113</td>
<td>133</td>
<td>65</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>Abnormal maternal serum screening</td>
<td>6</td>
<td>19</td>
<td>71</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Family history of genetic/chromosomal disorder</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Parental anxiety</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total No. (%) of abnormal cases</td>
<td>69 (12.4)</td>
<td>141 (25.3)</td>
<td>235 (42.1)</td>
<td>78 (14.0)</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>20 (3.6)</td>
<td>3 (0.5)</td>
</tr>
</tbody>
</table>

---
chromosomes. Indeed, the sensitivity dropped to 86.8% while the specificity remained at 99.9% (Table 4). Of 643 cases with an abnormal karyotype, there were 85 false-negative cases by FISH. The false-negative rate was 13.2%.

The FISH-negative cases included structural rearrangements, chromosome mosaicism, and trisomies of chromosomes other than the aforementioned (Table 5). While the FISH pick-up rate was 9.5%, the total abnormality rate by karyotyping was 10.9% (643/5883). Among cases that had various indications, including AU, there were 85 cases that were missed by FISH. The false-negative rate was 13.2%.

The FISH-negative cases included structural rearrangements, chromosome mosaicism, and trisomies of chromosomes other than the aforementioned (Table 5). While the FISH pick-up rate was 9.5%, the total abnormality rate by karyotyping was 10.9% (643/5883). Among cases that had various indications, including AU, there were 85 cases that were missed by FISH. The false-negative rate was 13.2%.

The introduction of RAS to prenatal diagnosis has alleviated many of problems associated with the long wait. Because aneuploidies of chromosomes 13, 18, 21, X and Y are the most frequent, the prenatal RAS FISH assay is centred on just these five chromosomes.

TABLE 3. Abnormalities detected by fluorescence in-situ hybridisation (FISH) versus karyotyping for chromosomes 13, 18, 21, X and Y

<table>
<thead>
<tr>
<th>Abnormal karyotype (for 13, 18, 21, X and Y)</th>
<th>Normal karyotype (for 13, 18, 21, X and Y)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal FISH (TP) 558 (FP) 1</td>
<td></td>
<td>559</td>
</tr>
<tr>
<td>Normal FISH (FN) 85</td>
<td>TN 5320</td>
<td>5324</td>
</tr>
<tr>
<td>Total</td>
<td>562</td>
<td>5321</td>
</tr>
</tbody>
</table>

* TP denotes true positive, TN true negative, FP false positive, and FN false negative
† Sensitivity (true positive rate) = 558/562 = 99.3%
‡ Specificity (true negative rate) = 5320/5321 = 99.9%
§ One false-positive case due to fetus with a balanced t(9;18)(p11.2;p11.1) resulting in a split 18 centromere
¶ Four false negatives due to suboptimal samples and technical problems

TABLE 4. Abnormalities detected by interphase fluorescence in-situ hybridisation (FISH) versus karyotyping for all chromosomes

<table>
<thead>
<tr>
<th>Abnormal karyotype</th>
<th>Normal karyotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal FISH (TP) 558</td>
<td>FN 85</td>
<td>643</td>
</tr>
<tr>
<td>Normal FISH (TN) 5320</td>
<td>5239</td>
<td>5240</td>
</tr>
<tr>
<td>Total</td>
<td>562</td>
<td>5321</td>
</tr>
</tbody>
</table>

* TP denotes true positive, TN true negative, FP false positive, and FN false negative
† Sensitivity (true positive rate) = 558/643 = 86.8%
‡ Specificity (true negative rate) = 5239/5240 = 99.9%

TABLE 5. Chromosomal abnormalities by various indications that were normal by fluorescence in-situ hybridisation

<table>
<thead>
<tr>
<th>Indication*</th>
<th>Balanced structural karyotype</th>
<th>Unbalanced structural karyotype</th>
<th>Mosaicism</th>
<th>Trisomies other than 13, 18, 21, X and Y</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced maternal age (≥35 years)</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Abnormal ultrasound</td>
<td>6</td>
<td>15</td>
<td>5</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>Family history of genetic/chromosomal disorder</td>
<td>6</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Abnormal maternal serum screening</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Parental anxiety</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other indications</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>34</td>
<td>13</td>
<td>8</td>
<td>85</td>
</tr>
</tbody>
</table>

* Excluding abnormal ultrasound indications, 8 cases had termination of pregnancy, 1 case ended in a miscarriage, 7 cases with malformations, 11 normal births, and 2 cases untraceable

Discussion

Conventional cytogenetic analysis of AF or chorionic villus tissues detects both chromosome aneuploidies and structural rearrangements with up to 99.5% accuracy. Owing to the need for cell culture however, a test may take anywhere between 1 and 2 weeks before it is completed due to the long culture time. Such long waiting period places significant emotional and psychological stress on patients and families. The introduction of RAS to prenatal diagnosis has alleviated many of problems associated with the long wait. Because aneuploidies of chromosomes 13, 18, 21, X and Y are the most frequent, the prenatal RAS FISH assay is centred on just these five chromosomes.

In this study, FISH using probes for chromosomes 13, 18, 21, X and Y detected 558

430 Hong Kong Med J Vol 16 No 6 December 2010 www.hkmj.org
abnormalities (Table 2). Trisomies 21, 18, monosomy X and trisomy 13 were the most frequent abnormalities in descending order. Among all the indications, an AU and MSS are the most critical in determining the requirement of a prenatal diagnosis.

The sensitivity and specificity of the RAS FISH assay versus karyotyping results were calculated to be 99.3% and 99.9%, respectively (Table 3). Of the 5883 cases, there were 558 true-positive cases, four false-negative cases and one false-positive case. The four false-negative results were due to technical problems one of the laboratories was facing at the time as well as poor sample quality. The single false-positive was due to a fetus with a balanced (t(9;18) (p11.2;p11.1)) rearrangement that resulted in a split chromosome 18 centromere, giving rise to three copies of chromosome 18 signals. One parent was found to carry the same rearrangement. The baby was born clinically normal. The results of the FISH assay are comparable to those reported in the literature and attest to the high specificity and sensitivity of the FISH assay in the detection of aneuploidies of chromosome 13, 18, 21, X and Y when compared with karyotyping, which is long considered the gold standard method of detection. When the detection rate of the RAS FISH was compared with karyotyping for all chromosome abnormalities, the sensitivity and specificity values were 86.8% and 99.9%, respectively (Table 4). Thus, when all chromosomal abnormalities were considered as opposed to just the five that were targeted, the specificity remained constant at almost 100% but the sensitivity had dropped by 12.5%. This difference was due to 85 false negatives (missed by FISH but detected by karyotyping). Among these, AU missed a total of 32 cases (Table 5). Excluding balanced structural rearrangements, there were a total of 55 unbalanced karyotypes with potential phenotypic abnormalities. Of these, an AU indication incurred the highest frequency with 26 (47%) cases compared with the other indications. Notwithstanding this finding, there were 29 abnormal cases in which there was a normal ultrasound. In these chromosomally unbalanced cases, abnormalities were detected only after birth or termination of pregnancy (TOP). Of these, family history of genetic/chromosomal disorder accounted for the highest number of cases (14), followed by AMA (11), MSS (3), and parental anxiety (1). This indicates that not only AU and MSS (Table 2) are important indicators of prenatal diagnosis, AMA and family history are also important. In the context of a normal ultrasound finding and MSS, offering RAS FISH as the sole genetic test with indications of AMA or FH will lead to underdiagnosis in certain circumstances.

Some of the above-mentioned 29 cases with normal ultrasound findings were likely to have been viable had the pregnancies been sustained. There were eight that underwent a TOP and one ended in a miscarriage. Two were lost to follow-up while the remainder went to term. Of those that went to term, seven were born with a wide range of malformations while the others were born clinically normal. Of the TOP cases and those born with malformations, the histopathological reports and neonatological findings of multiple congenital abnormalities were consistent with the features expected with the specific chromosomal abnormality. The chromosomal abnormalities of the TOP cases included imbalances due to deletions, partial trisomies due to adjacent-1 and 3:1 segregation patterns of reciprocal translocations, two cases of familial duplication of 3q syndrome due to insertions, and mosaicism for trisomies 12 and 14.

The total abnormality rate by karyotyping in this study was 10.9% (643/5883 cases) of which FISH detected over 91% (588/643). This figure is similar to the 11% abnormality rate reported by Dickinson et al,22 but much higher than the 4 to 4.8% rate obtained by Dupont and Carles24 and Leclercq et al.17 This apparent discrepancy may be due to our patient profile that more closely matched Dickinson’s patients22 in having a higher proportion assessed for MSS and AU and fewer for AMA,17 thereby resulting in a higher abnormality rate.

There is much reassurance for parents when a normal result is obtained by the RAS FISH assay, which is of primary importance. However, the final and conclusive result is not available until a full karyotype is obtained in the week following the FISH findings, in which abnormalities not detected by FISH can be unveiled. Indeed, evidence has been mounting that RAS is unable to replace conventional karyotyping owing to the number of misdiagnoses in the absence of ultrasound abnormalities.24,25 Several very large retrospective studies were carried out to address this issue. Thus, Caine et al20 looked at some 119528 AF samples and 23 077 chorionic villus samples and showed that stand-alone FISH missed around 1% of all prenatal chromosomal abnormalities, of which about a third might carried a significant risk of serious phenotypic consequences. Evans et al23 led an international multicentre collaborative assessment of 146 128 prenatal samples and determined that approximately 0.9% of abnormal karyotypes would be missed if only FISH were employed, notwithstanding the contribution of AU was not factored into the pick-up rates.

The evidence from this study shows that the strategy used by proponents of RAS FISH as a stand-alone test, and to use karyotyping only if there is an ultrasound abnormality, is unreliable. Many abnormalities will be missed. In this study, although the 18 cases with malformations (Table 5 footnote, including the 2 cases lost to follow-up) comprised only 0.3% of all the cases investigated by FISH and
karyotyping, institutions that call for FISH as a stand-alone test must seriously consider whether this is an acceptable risk.

Furthermore, to carry out RAS FISH, amniocentesis or CVS still needs to be performed. These invasive techniques carry an intrinsic risk of miscarriage of 0.5% and 1%, respectively. Small as such risks may be, it would seem prudent to have a thorough karyotype analysis to reveal the maximum information possible. However, if fetal cells or fetal DNA can be obtained by non-invasive procedures, screening by FISH as a stand-alone test may have a place. While the use of RAS if applied on a larger scale would lead to substantial economical savings, it nevertheless implies that there could be around a 1/500 to 1/1000 chance of missing a mentally and/or physically disabling chromosome disorder. There is also a debate as to whether the ensuing savings could outweigh the cost of health care of any corresponding children affected. Moreover, the cost of the FISH assay needs to be considerably reduced before it can be considered for large-scale screening.

Recently, array comparative genomic hybridisation techniques have been applied in prenatal diagnostic setting. Due to its ability to resolve microdeletions and microduplications down to 1 Mb and less, coupled with a genome-wide screen, this assay may have the potential to replace conventional cytogenetics in the future. It might delineate abnormalities with normal cytogenetics but where ultrasound abnormalities indicate a phenotypic genomic imbalance. Until array comparative genomic hybridisation becomes the mainstay diagnostic tests, we believe patients should opt for karyotyping instead of RAS as a stand-alone test.

Acknowledgements

The authors thank Ms Nicole Hackendahl and Ms Yim-leng Tham for their assistance in collating the data from the archives.

Declaration

No conflicts of interest were declared by the authors.

References

18. Leung TN. Rapid aneuploidy testing versus traditional karyotyping: is it better to know more? Hong Kong Med J 2008;14:4-5.