**ABSTRACT**

**Background:** Oestrogen/progesterone receptor expression in breast carcinoma is associated with good response to hormonal therapy and overall better prognosis. The predictive and prognostic capabilities of these receptors are enhanced by quantification of immunoreaction. There are several manual and automated methods for this purpose. Whether they yield comparable results that can be used interchangeably is not yet clear.

**Aim:** To compare the manual methods (H-score and Allred score) with automated methods (Immunoratio) for quantifying immunohistochemical (IHC) reaction for ER/PR in breast carcinoma.

**Materials and Methods:** Samples from established cases of breast carcinoma were processed and stained by immunohistochemical methods to demonstrate oestrogen receptor (ER) and progesterone receptor (PR). Receptor expression was quantified by manual methods (H-score, modified H-score and Allred score) and automated methods (basic and advanced Immunoratio). In modified H score, the intensity of reaction was assessed by measurement of mean grey value (H (MGV)) or optical density (H (DC-OD)) of deconvoluted image. The manual counting was done with cell counter plugin of Image-J (NIH). The scores were compared and Pearson’s correlation coefficient was determined.

**Results:** Both manual and automated methods produced results that were comparable. There was a statistically significant positive correlation among all methods (p<0.02). The strongest correlation was observed between advanced immunoratio and H (DC-OD) (p<0.001). Basic immunoratio appeared to be less reliable than the other methods. Staining intensity measurements by various methods did not significantly affect correlation. However, intensity measurements by optical density resulted in lower H-scores but led to more reliable detection of negative immunoreaction.

**Conclusion:** Both manual and automated methods of quantitation are comparable. Advanced immunoratio is a reliable alternative to manual methods. Cell Counter plugin is a useful tool for manual counting and quantitation.

**Keywords:** Allred score, Cell counter plugin, Deconvolution, ER, ‘H’ score, Image-J, Immunoratio, PR

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**INTRODUCTION**

The expression of steroid hormone receptors – oestrogen receptor (ER) and progesterone receptor (PR) – by breast carcinoma is associated with strong positive correlation with the latter’s response to hormonal therapy and overall prognosis [1]. Even without hormonal therapy, ER positive breast cancers have a better short-term prognosis than ER negative tumours [2]. Significance of PR expression is more uncertain. Its expression has a predictive value in assessing response to tamoxifen therapy in metastatic breast cancer [3]. Absence of PR expression in an ER-positive cancer (Luminal B type), makes the lesion more aggressive with greater genetic instability and increased mutations [4].

The predictive and prognostic value of receptor expression may be refined and improved if the assessment of receptors is semi-quantitative. Besides, American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) has recommended the use of image analysis for estimating the percentage of immunoreactive cells [5]. Following immunohistochemical demonstration of the receptor expression, they can be quantitated by several well established methods, both manual and automated. Some of the manual methods include H-score [6], immunoreactive Remmele score (IRS) [7] and the most popular Allred score [8]. One well-known automated method is “Immunoratio”, which is freely available online as basic or advanced protocols [9]. Whether the results of all the methods yield comparable values which can be used interchangeably is not yet clear. Shousha [10] suggested a conversion table to make H-score and Allred score more equivalent. But so far, the results of automated ‘Immunoratio’ have not been compared with those of the manual methods. In the present study, we have tried to find out if automated (Immunoratio) and manual methods (H score and Allred score) produce comparable values and whether they can be used interchangeably.

**MATERIALS AND METHODS**

Sixteen samples of tumour tissue from established cases of carcinoma of breast were fixed in buffered formalin within 30 min of their removal from the body and were fixed for at least 6 h (up to 24 h) before processing further for paraffin embedding. Three micron paraffin sections were cut using Leica Microtomes 2125 and 2245. One section was stained with haematoxylin and eosin. Other two sections were taken on poly-L-Lysine coated slides and were used for immunohistochemical (IHC) demonstration of oestrogen receptor (ER) and progesterone receptor (PR) using Novocastra™ Lyophilized Mouse Monoclonal Antibodies: NCL-ER-6F11 for ER and NCL-PGR for PR. Antigen retrieval was done using “High Temperature Antigen Unmasking Technique for Immunohistochemical Demonstration on Paraffin Sections” recommended by Leica Microsystems [11].

**Manual Scoring of ER and PR Immunostains**

Manual semi-quantitative assessment of ER and PR Immunostains were done by 2 methods: McCarty’s “H” score and Allred score. We used two Image-J tools to do the manual counting: Cell Counter and Point Tool [Table/Fig-1].
**H-score (6):** One hundred cells were counted sequentially and graded 0 to 3 depending on the intensity of staining (0 = negative; 1 = weak staining; 2 = moderate intensity; 3 = strong staining). The counting was done using “Cell Counter” plugin [Table/Fig-2] of morphometric software Image-J [12,13]. This plugin allows simultaneous categorisation and measurement. In conventional ‘H’ scoring (H-visual), the intensity was assessed visually. The final score was obtained by multiplying percentage of positive cells by the factor representing the intensity of staining (1 = weak; 2 = moderate; 3 = strong). As ‘0’ type is not available in Cell counter plugin, we used ‘4’ type as marker for ‘0’. The maximum score achievable is 300 [Table/Fig-3].

In conventional “H” score, the intensity of staining is assessed visually and is susceptible to subjective variations. In order to make intensity assessments more objective, we measured Mean Grey Value (MGV) and Optical density (OD). With cell counter plugin, this was achieved by clicking the ‘Measure’ button after selecting mean grey value in ‘Set Measurements’ option in “Analysis” menu. Using the values obtained, H-MGV was calculated.

**Automated scoring of Immunostains Immunoratio [16]**

Immunoratio is a freely available online image analysis tool based on Image-J. It involves colour separation by deconvolution, nuclear thresholding, particle segmentation and filtering, and calculation of ratio of DAB stained area to nuclear area [9]. Automated quantitative assessment of the immunostaining was done both by basic and advanced methods. The basic method does not allow user inputs/adjustments whereas advanced method allows background correction, image scale adjustment and colour threshold adjustment. The result is expressed as percentage positivity. The output is the montage of original IHC stain and the analysed image [Table/Fig-3] of H-visual. The intensity was assessed visually. The final score was obtained by multiplying percentage of positive cells by the factor representing the intensity of staining (1 = weak; 2 = moderate; 3 = strong). As ‘0’ type is not available in Cell counter plugin, we used ‘4’ type as marker for ‘0’. The maximum score achievable is 300 [Table/Fig-3].

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**To measure MGV using Point tool in Image-J, it was configured with the following options selected:** type – cross hair/circle; colour – red; size – extra-large; auto-measure – selected; overlay – selected; label points – selected. Auto-measure was set to measure the Mean Grey Value (MGV). The latter divided into quartiles (< 63.5, Strong; >63.5 – 127.5, moderate; >127.5- 191.5, weak positivity; >191.5-255, negative) was used as a measure of the intensity of staining.

As MGV was measured from RGB images, the value obtained is likely to be affected by the presence of other colours in addition to that of immunoreaction product. In an attempt to improve the accuracy of intensity measurement, we measured the intensity as optical density (OD) again on colour deconvoluted images. Colour Deconvolution was done using Colour Deconvolution plugin [14] for
samples giving much higher percentages of positivity compared to advanced method.

Pearson’s correlation matrix for all the methods used in the study is given in [Table/Fig-10]. There was statistically significant positive correlation among all the groups (p<0.02). However, there were subtle differences in the strength of correlation. Highest degree of correlation was observed between H-score (DC-OD) and Advanced immunoratio method (R=0.943; p<0.001). The conventional H-score (visual) showed greater correlation with the other conventional manual method, Allred score than with other methods (R=0.88; p<0.001). Among all, basic immunoratio method showed the weakest correlation with the conventional manual methods, H-score & Allred score (R=0.612 & 0.625; p = 0.01).

**DISCUSSION**

The role of determination of ER and PR expression in the predictive and prognostic assessments of breast carcinoma has long been established. More recently, the value of quantifying the receptor expression has been emphasised. This has led to introduction of several manual as well as automated methods for quantitating the receptor expression through image analysis. American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) has recommended the use of image analysis for estimating the percentage of immunoreactive cells [5]. At present, there are several well-established manual methods like H-score [6], IRS [7] and Allred score [8]. Immunoratio is the only free online automated image analysis tool for quantifying ER/PR [16]. It is also available as a plug-in for Image-J (NIH) [17].

*It is still uncertain whether the results obtained by these methods are comparable and can be interchangeably used. There has only been one attempt by Shousha [10] to make the two popular manual methods, H-score and Allred score, appear equivalent using a conversion table. So far, the manual methods and automated Immunoratio have not been compared. In the present study, we have tried to do just that. We have compared H-score and Allred score with basic and advanced protocols of automated Immunoratio. In addition, we used several modifications. In conventional manual methods, the staining intensity is assessed visually. This is susceptible to subjective variation. This may lead to inter-observer differences in the interpretation of results. To find out the extent to which the values may be affected, we made two pathologists to independently do manual H-score on all the slides. The results are given in [Table/Fig-10] (columns 2 and 3). Differences in the values between two observers varied from 0 to 82 (Sample 4 - 27%; average 33 or 11%). Similarly, if the same person did the score on a single sample repeatedly, we noticed similar variations unless the cells were marked each time and the same cells were counted. As Immunoratio is fully automated once it has been*
In order to eliminate subjectivity and make the intensity assessment more objective, we used mean grey value (MGV) or optical density (OD) of the immunoreaction as indicators of intensity of staining. Of the two, determination of OD on a deconvoluted image appeared to have subtle advantages. It can be adjusted to detect truly negative reaction much better than the other methods by careful calibration and selection of appropriate cut-off points. We used "Cell counter" plugin [12] of Image-J (NIH) [13]. We are not sure if this tool has been used for this purpose before as we could not find any documentation. This tool allows simultaneous counting, categorisation and measurement, and displays the result which can be saved in excel format for further statistical analysis.

In the present study, the scores obtained from all methods were comparable and showed significant positive correlation (R=0.591-0.943; p<0.001) [Table/Fig-10]. Two manual methods (H-score & Allred score) in which the intensity was visually assessed, showed stronger correlation with each other than with other more automated methods. Immunoratio in its basic mode, which allows for unattended result generation, also influence the quality of results.

The one question we need to answer is "is there a need for automated method if it is only as good as the manual method?" From our discussion above, it is fairly clear that automated method has the following advantages over manual methods:

- Immunoratio samples the whole photograph (all the tumour tissue in it) unlike manual methods in which only 100 cells are counted and the value is extrapolated for the rest of the image. So, using manual methods may lead to erroneous results due to sampling error; the sampling error is less likely with Immunoratio;
- It allows batch processing; since the measurement is automated, it allows for unattended result generation;
- Unlike manual methods which are time consuming, each run of immunoratio takes only a couple of minutes;
- It produces consistent, reproducible and repeatable results;
- It generates an informative pictorial montage aseport;
- It is free and readily available on the web. For off-line use, Image-J plugin is available.

CONCLUSION

In conclusion, quantitation of ER/PR expression by both manual (H-score and Allred score) and automated (basic and advanced Immunoratio) methods produced comparable results. There was statistically significant positive correlation among the various methods. Advanced Immunoratio appears to have a good correlation with the manual methods. But it needs to be carefully adjusted particularly with regard to brown and blue colour thresholds. Once it is properly tuned, it can produce results that are comparable to more established manual methods and appears to be a reliable and hassle free alternative to the time consuming manual methods. But basic immunoratio is less satisfactory. For manual counting and quantifying, Cell Counter plugin of Image-J (NIH) appears to be a very effective tool. Quantifying the intensity of immunoreaction is made more objective by OD determination on deconvoluted image.

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REFERENCES

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