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Estrogen and Progesterone Receptor Assays in Human Breast Cancer: Sources of Variation Between Laboratories

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Abstract—*The importance of cytosol preparation as a source of inter-laboratory variation in estrogen (ER) and progesterone (PR) receptor measurements was evaluated together with protein measurements and receptor assays for five laboratories in Sydney, Australia, using pooled, fragmented human breast cancer samples. Protein measurement was only a minor source of variation between the laboratories with a CV of* 13%. *For ER measurements, sources of variation due to either assay or cytosol preparation methods contributed* 40 *and* 39% *CV each. However, the variation due to assay method was reduced to* 25% *CV when the results from one laboratory with a known effect of a different ER assay protocol were excluded, suggesting that assay standardization could readily reduce this source of variation. In contrast, the source of a large cytosol error* (39% *CV) could not be identified. Variations in PR results were similar to ER, but the sources could not be accurately estimated. It is concluded that cytosol preparation as well as assay methods were major sources of between laboratory variation and need to be further investigated and standardized. This approach should reduce these sources of variation since it was found that the within laboratory cytosol and assay variations were only* 13 *and* 18% *CV, respectively, for the ER measurements.*

INTRODUCTION

It is now accepted that in human breast cancer, the response to endocrine therapy can often be predicted by the presence of estrogen receptors (ER) [1, 2] and progsterone receptors (PR) [3] in human breast cancer. However, it is also well known from quality assurance (QA) trials that large inter-laboratory variations exist for steroid receptor assays [4–9]. The main aim of these trials has been to standardize the assays, and although large variations have been demonstrated for both the cytosol protein and receptor assays, little attention has been focused on the methods of cytosol preparation as a source of variation between laboratories.

Five laboratories in Sydney, Australia, agreed to investigate the sources of variations between their ER and PR assays in a QA trial. The design of the QA trial made it possible to distinguish between cytosol preparation, protein measurement and receptor asays as sources of variations in ER and PR measurement between laboratories. This was achieved by comparing the results obtained within and between the participating laboratories with those obtained by the reference laboratory for the cytosols prepared by the participants.

MATERIALS AND METHODS

Experimental design

ER and PR positive primary breast cancer specimens which had been stored for up to 3 months at −70° C were collected and pooled by the reference laboratory. The specimens were snap-frozen in liquid nitrogen, and finely divided tissue fragments were prepared by percussion between two steel blocks cooled in liquid nitrogen. The pooled tissue fragments were carefully mixed, and standard

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samples were randomly assigned. Four samples were immediately sent on solid CO_2 to each of the participating laboratories, and all laboratories started their cytosol preparations at a given time 2 hr after sample preparation. All laboratories divided each of their four cytosols in halves. One half was retained by each laboratory for immediate ER and PR assays while the second half was immediately returned on ice to the reference laboratory. The participant cytosols were received within 1 hr of preparation and immediately assayed for ER and PR by the reference laboratory together with its own samples. All the reference laboratory assays were performed in ignorance of the laboratory source of cytosols. Thus, the source of variation due to between laboratory protein measurement and ER and PR methodology is eliminated by the provision of having the reference laboratory assay the participant cytosols. In contrast, the results obtained by the participants include between laboratory variation due to all three sources. By comparing the results obtained by the reference laboratory and participants for the same cytosols, the sources of variation could be quantitated.

ER, PR and protein assays

The homogenization buffer for cytosol preparation consisted of 10 mM Tris–HCl, 1.5 mM EDTA, 1 mM mono-thioglycerol and 10% (v/v) glycerol at pH 7.4 [9]. The other details of the ER, PR and protein assays, although similar, were according to each laboratory's routine methodologies. Thus, all the participants used Lowry's method of protein measurement, multiple point ER and PR assays, charcoal to separate free and bound ligand and Scatchard and/or Woolf plot analysis of the results. However, in contrast to the other participants, laboratory A used methylcellulose coated charcoal (MCC) instead of dextran coated charcoal (DCC) to separate free and bound ligands. It should also be noted that laboratories B, C and D used "sheering" methods to homogenize the tissue fragments. Laboratories B and D used the "Polytron", and laboratory C the "Ultraturrax". In contrast, laboratory A and reference laboratory used the "Microdismembrator" to pulverize the tissue fragments for cytosol preparation. The approximate ratios of tissue weight (g) to homogenization buffer (ml) were 1 : 10 for laboratories A, B and D, and 1 : 6 for laboratory C and reference laboratory. The participants were asked to treat the samples of tissue fragments as ordinary tissue specimens as far as possible.

Statistical methods

A random effects model [10] was used on the data with respect to reference laboratory results, participant results, and the sum and difference of reference laboratory and participant results matched for sample. This analysis allowed quantitation of the various components of variation between laboratory cytosol preparation difference (L), within laboratory variation in cytosol preparation from sample to sample (C), between laboratory variation in receptor assays (A), and within laboratory receptor assay variation (E).

The relationship between these quantities was expressed as follows:

$$X_{ij1} = \mu_{ref} + L_i + C_{ij} + E_{ij1} \quad (1)$$

$$X_{ij2} = \mu_i + L_i + C_{ij} + E_{ij2} \quad (2)$$

where μ_{ref} and μ_i are the mean results produced by the reference and participating laboratories, respectively, and X_{ijk} is the result produced by the reference laboratory ($k = 1$) or the participant laboratory ($k = 2$) on cytosol prepared by the ith laboratory for its jth sample.

Taking the difference between Eqns (1) and (2):

$$D_{ij} = X_{ij2} - X_{ij1} = \mu_i - \mu_{ref} + E_{ij2} - E_{ij1}. \quad (3)$$

Summarizing Eqns (1) and (2):

$$S_{ij} = X_{ij2} + X_{ij1}$$

$$= \mu_i + \mu_{ref} + 2L_i + 2C_{ij} + E_{ij1} + E_{ij2}. \quad (4)$$

One-way analysis of variance (ANOVA) can be carried out on the variables described in the above equations to derive the parameters of interest. If it is assumed that E_{ij2} and E_{ij1} are from the same population, E, then ANOVA on D_{ij} [Eqn. (3)] has a mean square residual of $2\sigma_E^2$, whereas ANOVA on S_{ij} [Eqn. (4)] has a residual of $2\sigma_E^2 + 4\sigma_C^2$. ANOVA on X_{ij1} [Eqn. (1)] gives a mean sum of squares for between laboratory differences of $\sigma_E^2 + \sigma_C^2 + m\sigma_L^2$ where m is a constant depending on the number of replications, and the mean square residual is $\sigma_E^2 + \sigma_C^2$. If the difference between laboratories is taken as a random laboratory effect, the mean square for differences between laboratories from ANOVA on D_{ij} [Eqn. (3)] is $m\sigma_A^2 + 2\sigma_E^2$ where m is again a constant depending on the number of replications. Subtraction between the various sums of the squares thus calculated enables the contribution of the sources of variation to be quantified. Appropriate variance ratios (F values) were used to calculate significance.

To stabilize the variance for ANOVA, a logarithmic transformation was used with ln(1 + ER) and ln(1 + PR) as the transformed variable. The standard deviations (S.D.) of natural logs when multiplied by 100 is then approximately the same as the % CV of the raw data. Because of this transformation, zero values had to be excluded. However, for the ER assay, the only zero value excluded (laboratory A, Table 2) was also an obvious outlier. The remaining ER values for laboratory A were 94,

Table 1. *Results of protein measurements* (mg/ml *cytosol*)

	Source of cytosol (laboratory)				
	A	B	C	D	Ref
Ref	1.87 (16)	2.30 (5)	3.84 (8)	2.05 (14)	3.73 (17)
Part	1.90 (31)	1.83 (9)	4.30 (8)	2.10 (11)	3.73 (17)

The mean values and CVs (%) are shown for the participants A to D and the reference laboratory. Ref: cytosols prepared by participants have been measured by the reference laboratory. Part: cytosols prepared by the participants have been measured by the participant.

60 and 90 fmoles per mg protein. The zero values obtained by the reference laboratory for PR assays, Table 3, prevented much use being made of the calculated sources of variation and highlighted the instability of PR assays (see Results and Discussion sections for PR assays). The components of variance of the ER and PR measurements presented (Table 4) are those calculated from the log transformed data excluding zeroes, as this seems to best fulfil the requirements for ANOVA and therefore provide the most realistic variance data. However, in order to ensure that our conclusions were not artefacts of the transformation, we also carried out the analysis on all the raw data (including zero values), as well as including and excluding various laboratories. None of these manoeuvres seriously altered the conclusions except in one instance which will be discussed. In contrast, for protein assays, the raw data was used to calculate the reported components of variance.

RESULTS

Protein assay

Overall mean reference laboratory and participant results were not significantly different. The mean protein concentrations and CV for each laboratory are shown in Table 1, and ranged from 1.8 to 4.3 mg protein/ml cytosol and 5–31% CV, respectively. The % CVs for the different sources of variation are given in Table 4. The within laboratory assay variation in protein measurement was 6% CV and the between laboratory variation was 13% CV. Cytosol variation for protein includes the variation due to different reconstitution volumes and protein uptake into solution. The within laboratory variation due to this source was 10% CV and the between laboratory variation was 34% CV.

ER assay

The results of the ER assays are shown in Table 2. In the first column are presented the ER values obtained by the reference laboratory for the cytosols prepared by the participants, where the mean ER values ranged from 37 to 111 fmol/mg protein, and the CVs from 17 to 50%. In the second column are the ER values obtained by the participants for their own cytosol preparations, where the mean ER values ranged from 26 to 153 fmol/mg protein, and the CVs from 13 to 50%.

As for protein assays, overall mean reference and participant results were not significantly different. Individual laboratory results are given in Table 2, and % CVs for the sources of error in Table 4. The within laboratory variation due to ER assay was

Table 2. *Results of ER assays*

Sources of cytosol	ER (fmol/mg protein)		K_d (nM)	
	Ref	Part	Ref	Part
A	81* (23)	153 (38)	0.33 (110)	0.71 (151)
B	111 (22)	111 (32)	0.51 (76)	0.11 (25)
C	49 (17)	55 (27)	0.37 (33)	0.06 (23)
D	37 (19)	26 (13)	0.14 (70)	0.14 (83)
Ref	65 (50)	65 (50)	0.42 (100)	0.42 (100)

*Excludes one zero ER value.
The mean values and CVs (%) are shown for the participants A to D and the reference laboratory. Ref: cytosols prepared by participants have been assayed for ER by the reference laboratory. Part: cytosols prepared by the participant have been assayed for ER by the particpant.

Table 3. *Results of PR assays*

Sources of cytosol	PR (fmol/mg protein)		K_d (nM)	
	Ref	Part	Ref	Part
A	47 (2)	137 (26)	0.41 (65)	0.19 (0)
B	35 (71)	69 (33)	1.18 (90)	1.21 (35)
C	0* (0)	54 (5)	— (—)	0.33 (9)
D	20* (112)	69 (27)	0.80 (88)	0.70 (51)
Ref	25 (52)	25 (52)	0.38 (65)	0.38 (65)

*Excludes four and two zero values in C and D, respectively.
Abbreviations, symbols and values have been explained in Table 2.

Table 4. *Components of variance influencing discrepancies in protein, ER and PR assays*

Sources of variation	Protein	ln(1+ER)	ln(1+PR)
Within lab assay method	6	18	56
Between lab assay method	13*	40*	—
Within lab cytosol method	10*	13	22
Between lab cytosol method	34*	39*	29

For protein, % CVs are given. For ER and PR approx. % CVs obtained by multiplying standard deviations of the ln parameters by 100 are given.
*Significantly different from zero ($P < 0.005$).

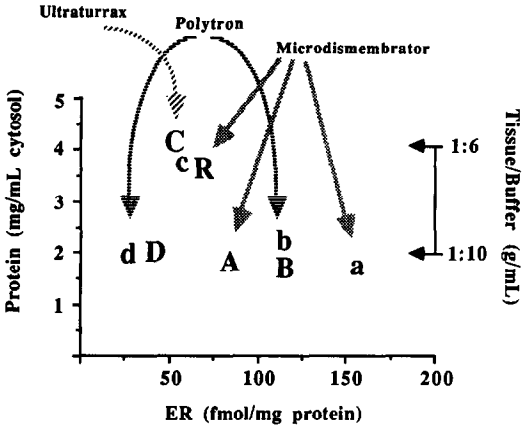

Fig. 1. Comparison of mean protein and ER measurements obtained by particiants (capital letters) and reference laboratory (small letters) as correlated with cytosol preparation methods (homogenization equipment and approximate tissue/buffer ratio).

18% CV while the between laboratory variation due to this source of error was 40% CV. If laboratory A (different assay methodology) was excluded from the analysis, the between laboratory variation due to ER assays was reduced from 40 to 25% CV. The within laboratory cytosol preparation variation was then only reduced from 18 to 14% CV.

The between laboratory variation due to cytosol preparation was 39% CV, and was little altered by the inclusion or exclusion of laboratory A. These results were calculated on ER per mg of protein, and the protein concentrations derived by the reference laboratory were used to eliminate protein assay variation in ER assays as a source of error. However, the use of individual protein results from the participants made little difference to the results as expected from the small between laboratory variation (13% CV) for protein assays.

Table 2 also shows a comparison of the K_ds obtained by the reference laboratory and the participants. The mean K_ds ranged from 0.14 to 0.51 nM and from 0.06 to 0.71 nM for the reference laboratory and participants, respectively, but no correlation between ER values and K_ds were found.

The ER assay results are compared with cytosol preparation method in Fig. 1. As may be expected, Fig. 1 shows that those laboratories which used a higher tissue to buffer ratio produced cytosols with higher protein concentration. In contrast, the homogenization equipment used (Ultraturrax, Polytron or Microdismembrator) had no obvious effect on either protein or ER concentration in the cytosols.

PR assays

The results of the PR assay are shown in Table 3. The mean PR values obtained by the reference laboratory ranged from 0 to 47 fmol/mg protein, and the CVs from 0 to 112%. The mean PR values obtained by the participants ranged from 25 to 137 fmol/mg protein, and the CVs from 5 to 52%. Apart from the great variation in results, another important aspect of the PR results was that, on average, participant PR results were not the same as reference laboratory results ($P < 0.005$). Thus, the participant results were 1.9 times the reference laboratory results. Similarly, excluding zero results in Table 3, the within laboratory group CV of reference laboratory results was 71% on average, while for the participants it was only 25%. These differences prevented much use being made of the calculated sources of variation shown in Table 4. However, one-way ANOVA showed that cytosol preparation contributed 29% CV to the variation between laboratories (Table 4).

Table 3 also shows a comparison of the K_ds obtained by the reference laboratory and the participants. The mean K_ds ranged from 0.38 to 1.18 nM and from 0.19 to 1.21 nM for the reference laboratory and participants, respectively, but as for the ER assays, no correlation between PR values and K_ds were found.

DISCUSSION

Design of QA trial

None of the five laboratories participating in the QA trial used identical methodology for measurement of either ER or PR. The trial was designed, therefore, so that differences in results could be compared through the reference laboratory rather than attempting to correlate differences in methodology with differences in results.

The procedure for ER and PR assays can be divided in four parts: (1) collection, storage and transport of specimens; (2) cytosol preparation; (3) protein assay; and (4) ER and PR assays including analysis of results. The effect of differences in collection, storage and transport of specimens on ER and PR results were standardized and therefore not investigated in this trial.

Two previous series of QA trials have attempted to distinguish between the contribution of differences in cytosol preparation and in ER assays on the results [4, 5]. In one series, trials were conducted with either pulverized tissue or freeze-dried cytosol from human breast cancer [4]. Although some improvement was obtained in the average CV for the ER results between laboratories using freeze-dried cytosol (from about 95% down to 60%), the differences between duplicate samples within laboratories were greater with freeze-dried cytosols than with powders. In this study [4], a 3-fold difference in protein measurements was found between laboratories, and it was suggested that the differences in protein values contributed more to the differences in ER results than the methods of cytosol preparation.

The other series of QA trials [5] used human breast cancer specimens as standard samples, and attempted to correlate the ER results obtained by the participants with differences in cytosol preparation methods. Although breast cancer tissue was found not to be suitable as standard samples for quantitative ER measurements, it was suggested that differences in thiol-reagent content in assay medium and methods of homogenization yielded discrepancies in the qualitative ER measurements (positive vs. negative) between the laboratories.

Other QA trials have used either pulverized tissue specimens or freeze-dried cytosols as standard samples, and have not attempted to investigate the relative contribution of differences in cytosol methodology on the variation in ER and/or PR measurements [6–9].

Cytosol preparation

The difference in tissue to buffer ratios used to prepare cytosol between laboratories (Fig. 1), accounted for the higher protein concentrations obtained in cytosols prepared by laboratory C and reference laboratory compared to laboratories A, B and D.

The variation in ER concentration could not be accounted for by differences in the type of homogenization equipment used (Fig. 1), and we suggest that it must be due to operating factors affecting the risk of receptor degradation and efficiency of receptor extraction.

Protein assay

Previous QA trials in Australia have found up to a 3-fold difference in protein measurements between laboratories [4]. It is therefore reassuring to note that in the present QA trial the within and between laboratory assay variations were small (6% and 13% CV, respectively, Table 4), and were not a major component of discrepancies in ER assays.

It is important to note that the between laboratory protein concentrations only ranged from 1.9 to 4.3 mg/ml cytosol (Table 1) resulting in only 34% CV (Table 4), and were in the optimum range for steroid receptor assays [11, 12].

ER assay

The source of variation between ER assays due to assay methodology for the five participating laboratories were large (40% CV, Table 4). The source of some of this variation can be identified. Laboratory A used MCC instead of DCC to separate bound and free ligands, and it is known that MCC produces about twice as high ER and PR results as compared to DCC separation [13]. Excluding laboratory A, the between laboratory CV due to ER assay for the remaining laboratories was reduced from 40 to 25%.

The importance of variations in cytosol preparations are obvious from Table 2 where the provision of having one laboratory carry out all the assays does little to remove between laboratory variation. Results are still significantly different between laboratories ($P < 0.005$). The magnitude of the variation due to cytosol preparation was 39% (Table 4), and from the assay protocols there were no obvious causes of this variation although the cytosol preparation protocols varied considerably. Variation in reconstitution and the volumes used were corrected for by expressing results in terms of protein and variation in the composition of the samples provided to the participants had only a small effect as the within laboratory CV due to cytosol preparation was only 13%.

PR assay

The results showed that both cytosol preparation and assay methodologies were substantial sources of discrepancies in PR values between laboratories (Tables 3 and 4). Furthermore, in some instances the reference laboratory could not detect PR in the cytosols prepared by the participants (Table 3), and its PR values were significantly lower ($P < 0.005$) than the participants'. It should be noted that the reference laboratory started its PR assay about 1 hr later than the participants due to the time involved in transport of cytsols from participant laboratories to the reference laboratory.

It is suggested that loss of PR binding capacity is the source of the lower PR values obtained by the reference laboratory. This is supported by the results from a separate series of experiments using samples from the same pool of breast cancer specimens as in this study. It was found that storage of these cytosols at 4° C for 1 hr may result in 0 PR values, and that addition of sodium molybdate to the cytosol buffer prevents the loss of PR binding capacity with time [14]. Although molybdate prevents loss of both ER and PR with time [14], it should be noted that molybdate may also increase the yield of cytosolic ER which cannot fully be accounted for by the accompanying loss of nuclear ER [15].

It is concluded that both cytosol preparation and PR assays need to be standardized, and that sodium molybdate should be incorporated in order to obtain reliable and reproducible PR measuremnts between laboratories. For both ER and PR measurements it is essential to identify the causes of variation between laboratories, such as DCC or MCC separation of bound and free ligands, otherwise one ends up with "controlled assays", but with no real idea of the absolute value of a receptor concentration.

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