Eur J Cancer Clin Oncol, Vol. 25, No. 7, pp. 1087–1093, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Sources of Improvement in Between Laboratory Variation in Estrogen and Progestin Receptor Measurements using Tissue Samples during the Australasian Quality Assurance Programme

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Abstract—*Tissue and cytosol samples were compared as quality control material for assessment of between and within laboratory error in measurement of estrogen and progestin receptors (ER and PR) in a series of four trials for a total of* 17 *participating laboratories during the Australasian Quality Assurance Programme. For tissue samples, a substantial reduction in between laboratory CVs for both ER and PR from about* 90 *to* 50% *was achieved during the programme. In contrast, for cytosol samples a substantially lower between laboratory CV of about* 30% *was obtained. Tissue sample heterogeneity could be excluded as a major source of variation between laboratories. The likely source of the observed improvement in CV for tissue samples during the trials was due to a reduction of the initial under-estimation of receptor concentration in tissue samples by some of the participants. Although cytosol preparation from tissue samples was shown to be one major source of error, other sources of error such as the receptor assay itself and the associated protein measurements were identified. It is concluded that fragmented tissue samples are essential for a realistic assessment of between laboratory error in receptor measurements in biopsy material such as obtained from clinical breast cancer samples.*

INTRODUCTION

ESTROGEN and progestin receptor (ER and PR) assays are widely used as diagnostic tools to predict clinical response to endocrine therapy in human breast cancer. The presence or absence of receptors in breast cancer biopsies are frequently used for this purpose, but it has been reported that the quantitative measurement of receptors provides an improved correlation with response to endocrine therapy [1]. Regardless of which approach is used, quality assurance of receptor assays between and within laboratories is essential, and several national and international quality assurance programmes [2–8] have aimed at reducing the between laboratory variation in quantitative receptor measurements. Due to inherent problems with stability, availability and homogeneity of tissue samples for receptor measurements, cytosol samples prepared by a reference laboratory have generally been used as quality assurance material. Although cytosol samples are suitable for testing and standardizing

Accepted 9 March 1989.
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the receptor and associated protein assays, this type of material does not allow evaluation of the participants' ability to prepare high quality cytosol samples from tissue specimens such as breast cancer biopsies. We have previously shown that cytosol preparation may be a major source of between laboratory error in receptor measurements, suggesting that tissue samples should be used as quality assurance material for receptor assays where cytosols are prepared from solid tissues [9]. Furthermore, it has been reported that, even within one laboratory, the main source of between assay variation is the cytosol preparation [10], confirming that cytosol samples have only limited value as quality assurance material for receptor assays of tissue specimens.

The principal aim of the Australasian Quality Assurance (AQA) Programme reported here was to reduce between laboratory error over a 2 year period by alerting the individual participants to any discrepancies in their results as compared to the other participants after each trial. The programme facilitated communication between laboratories at workshops held at the annual conferences of the Australian Association of Clinical Biochemists, and one of the longer term aims of the programme was to arrive at generally acceptable standardized ER and PR assays.

The purpose of this paper is to evaluate the usefulness and necessity of tissue samples as quality control material. Fragmented tissue samples in addition to cytosol samples were therefore used as a realistic quality assurance material for evaluation of between laboratory variations in ER and PR measurements.

MATERIALS AND METHODS

Five distributions of quality control material were made. On each occasion the participants received four identical samples of tissue fragments and/or lyophilized cytosols for ER and/or PR assays. The samples were allocated at random, and the participants did not know that each set contained four identical samples. Tissue samples were prepared from calf uteri which were fragmented into 1–2 mm pieces by percussion between two steel blocks at liquid nitrogen temperature. In order to adjust ER and PR concentration to a realistic range of about 100–200 fmol/mg protein for optimum quantitation and qualitative classification as well as minimum between trial variation, bovine minced meat was also fragmented and then mixed with the calf uterus preparation.

The fragmented tissue preparation was stored at −70°C for up to 1 week prior to distribution to the participants. However, tissue samples prepared in this way were found to be stable for at least 2 months at −70°C (unpublished observation). On the day of sample distribution, about 1 g of fragments was prepared per tissue sample. The samples were transported to the participants on dry ice within 24 h and then stored at −70°C or in liquid nitrogen until assay. In two of the trials, standard lyophilized cytosol samples prepared in Tris buffer containing molybdate and glycerol at pH 7.4 were also distributed to the participants under the same storage and transport conditions as for tissue samples. However, in the June 1986 trial, calf uterus only was used as material for cytosol preparation, whereas in the May 1987 trial, the cytosol samples were prepared by the reference laboratory from the mixed tissue sample material described above. It should also be noted that for the May 1987 trial the participants were requested to homogenize the tissue samples in the same buffer as used by the reference laboratory for preparation of the cytosol samples (10 mM Tris, 1.5 mM EDTA, 5 mM molybdate and 1 mM monothioglycerol, pH 7.4). This strategy ensured that not only were the tissue and cytosol samples prepared from identical material, but also that any errors in protein measurements due to differences in buffer composition between tissue and cytosol samples were avoided [11]. The individual laboratories reported within assay ER and PR concentrations for each set of tissue and cytosol samples using their own In-House radioreceptor assay methods. The laboratories used different methods of tissue homogenization (Microdismembrator, Ultraturrax or Polytron), but all used charcoal to separate free and bound ligands as well as similar buffer compositions.

We have previously found [9] and recently confirmed (unpublished results) that the type of homogenization method cannot be distinguished as a source of between laboratory error. Nonetheless, the participants were still encouraged to standardize this and all other aspects of their assays during the AQA Programme. Thus, the importance of including molybdate in the homogenization buffer to prevent loss of measurable receptor concentration for both ER and PR [12], the desirability of standardizing the method of protein measurement in cytosols [13], the EORTC recommendation of using the Microdismembrator for tissue homogenization to minimize variability of cytosol preparation [14], and the possible advantage of using gelatin [15, 16] or methylcellulose [9] to coat charcoal for separation of free and bound ligands, were all topics for communication and discussion during the programme. However, any assay changes within and between individual laboratories during the programme were not evaluated, since the large number and permutations of all possible assay parameters, as well as variation in technical proficiency, precluded this approach. Instead, the principal aims were to evaluate the usefulness of tissue samples as quality control

material and to monitor the progress in reducing the between laboratory CV of receptor measurements during the programme as a result of 'trouble-shooting' by individual laboratories.

In order to monitor progress of receptor measurements by the participants, the average within laboratory CV was calculated as the root mean square of the individual laboratories' CV. Between laboratory CV was calculated as the CV of the means for the laboratories, and the overall CV as the CV of all the data.

RESULTS AND DISCUSSION

The individual laboratories' ER results for tissue as well as cytosol samples over a 2 year period are shown in Table 1. It can be seen that large variations of ER concentration were obtained within each trial. For example, in the first trial with tissue samples in April 1985, the mean ER concentration ($n = 4$) obtained by the participants ranged from 0 to 160 with an overall mean value of 51 fmol/mg protein and a CV of 105%. However, the overall CV was reduced to 62% in the last trial with tissue samples in May 1987 (see Table 1). In contrast to tissue samples, cytosol samples resulted in less variation in ER measurements with overall CVs of only 34 and 41% (see Table 1).

Large between laboratory variations were also observed for the PR assays as shown in Table 2. For tissue samples, mean PR concentrations for individual laboratories ranged from 2 to 483 (overall mean 147) in the April 1985 trial, but as for the ER assays, the overall CV decreased during the programme from 92 to 66%, and similarly a lower CV was obtained for cytosol as compared to tissue samples (25% vs. more than 66%, Table 2).

Several factors, in addition to improved receptor assays, could have contributed to the reduction in CV for tissue samples between trials, and to the lower CV obtained for cytosol as compared to tissue samples. For example, differences in receptor concentration in samples between trials, a greater heterogeneity of tissue vs. cytosol samples, as well as the additional source of error for tissue samples due to cytosol preparation, may all have contributed to the improvements in CV. However, the overall mean receptor concentrations for tissue samples were not significantly different between trials, although individual laboratories means may have changed relative to the other participants. Furthermore, for ER in particular, the same material was used to prepare both tissue and cytosol samples by the reference laboratory in the May 1987 trial (see Materials and Methods), where the overall CVs were 62 and 41% respectively for the two types of samples. It is therefore unlikely that any differences in receptor concentrations between trials contributed to the observed improvement in CV during the programme, or to the differences in CV between tissue and cytosol samples.

The effect of sample heterogeneity on the relatively high overall CV obtained for tissue samples, may be evaluated by comparing between and within laboratory CVs as shown in Figs. 1 and 2 for

Table 1. *Mean ER* (fmol/mg *protein*) *measurements* (n = 4) *by individual laboratories in the AQA programme from* 1985 *to* 1987. *Within laboratory % CVs are given in parentheses*

Date Sample Lab I.D.	Apr 1985 Tissue	Sept 1985 Tissue	Feb 1986 Tissue	May 1987 Tissue	June 1986 Cytosol	May 1987 Cytosol
1	66 (25)	130 (16)	40 (24)	46 (32)	—	234 (14)
2	32 (76)	163 (26)	55 (42)	212 (28)	268 (5)	229 (4)
3	30 (36)	—	100 (31)	224 (27)	322 (9)	220 (8)
4	8 (200)	29 (68)	—	—	—	—
5	28 (100)	143 (27)	56 (46)	203 (18)	254 (11)	231 (16)
6	8 (200)	4 (116)	58 (50)	75 (14)	166 (18)	71 (3)
7	160 (40)	119 (10)	108 (14)	—	494 (30)	—
8	137 (34)	143 (13)	63 (17)	116 (34)	287 (11)	216 (2)
9	46 (27)	91 (52)	44 (16)	225 (39)	414 (4)	209 (9)
10	114 (29)	147 (41)	104 (66)	82 (13)	389 (15)	188 (11)
11	51 (87)	—	—	53 (76)	240 (33)	128 (12)
12	18 (102)	—	—	—	—	—
13	5 (40)	42 (33)	38 (31)	129 (20)	265 (9)	137 (9)
14	—	30 (41)	76 (17)	76 (30)	248 (9)	112 (18)
15	0 (—)	24 (47)	6 (30)	106 (52)	296 (51)	106 (27)
16	94 (20)	115 (17)	—	129 (8)	291 (21)	138 (6)
17	27 (42)	28 (28)	—	29 (8)	188 (3)	38 (15)
Mean	51 (105)*	86 (71)	62 (61)	122 (62)	294 (34)	161 (41)

*Overall % CV.

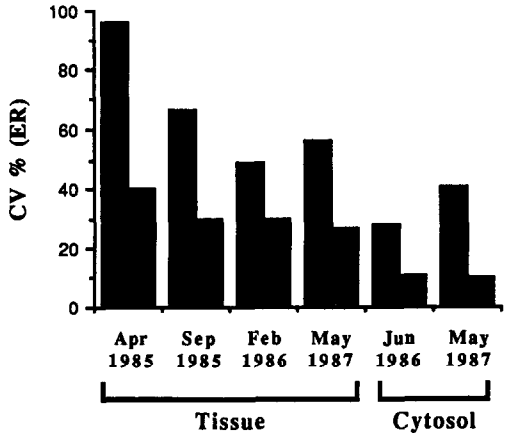

Fig. 1. Changes in between (filled bars) and within (hatched bars) laboratory CVs for ER measurements of tissue and cytosol samples during the AQA Programme.

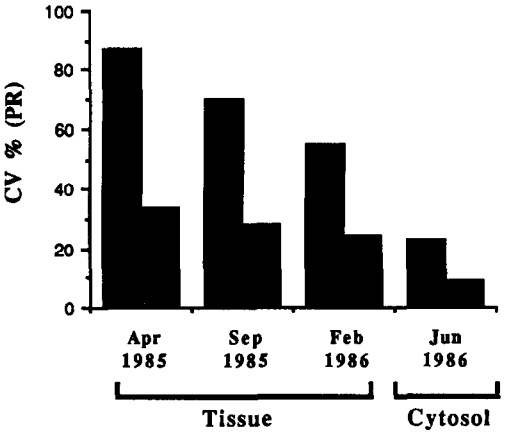

Fig. 2. Changes in between (filled bars) and within (hatched bars) laboratory CVs for PR measurements of tissue and cytosol samples during the AQA Programme.

ER and PR respectively. Heterogeneity of tissue samples may be a major component of within laboratory CV obtained from individual sample results. However, the within laboratory CV is always substantially lower than the between laboratory CV, suggesting that additional sources of error, such as cytosol preparation, receptor assays and associated protein measurements, are the main components of between laboratory CV for tissue samples. Note also that these additional sources of error for tissue samples, as indicated by the between laboratory CV, decreased during the programme and approached those obtained for cytosol samples, suggesting improvement in sources of error unrelated to sample heterogeneity.

It can also be seen from Figs. 1 and 2 that the within laboratory CV for tissue samples were much higher than for cytosol samples (about 30% compared to 10%). The additional components of variation for assay of fragmented tissue samples compared to cytosol samples are tissue heterogeneity and cytosol preparation. It has previously been reported for a single laboratory [10] that even for the same batch of pulverized tissue samples, cytosol preparation was the main source of error. Within laboratory CVs of about 35% for pulverized tissue and about 10% for cytosol samples were reported, which are comparable to the CVs obtained in the AQA Programme. It may therefore be concluded that tissue sample heterogeneity was not a major component of between laboratory CV, and probably not for within laboratory CV either.

In order to illustrate the contribution by individual laboratories towards the improvement of CVs during the programme, the participants were ranked according to their results in the April 1985 trial. Relative changes in mean individual results between trials could then be compared with the other participants, since the overall mean receptor concentrations were not significantly different between trials. Some relative changes have been shaded to illustrate trends in Figs. 3 and 4. It is apparent that within the group of participants that initially obtained low results, there appeared to be an increase in ER and PR during the programme compared to the rest of the participants, which would have contributed to the reduction in between laboratory CV. This reduction in CV may perhaps

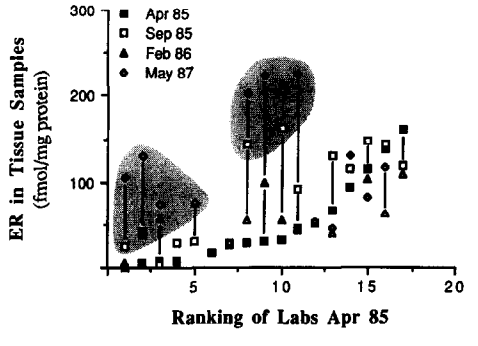

Fig. 3. Improvement in ER measurements during the AQA Programme for individual laboratories compared to the April 1985 trial. The shaded areas represent laboratories where the results showed apparent changes relative to the other participants.

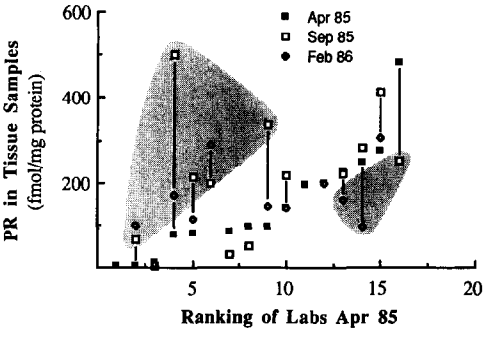

Fig. 4. Improvement in PR measurements during the AQA Programme for individual laboratories compared to the April 1985 trial. The shaded areas represent laboratories where the results showed apparent changes relative to the other participants.

also have been enhanced by the relative decrease in some of the highest PR results (see Fig. 4). It was also noted that, for the May 1987 trial, there appeared to be a second group of four laboratories that changed from a relatively medium to high ER result (see shaded area in Fig. 3). The relative changes in receptor measurements for individual laboratories shown in Figs. 3 and 4 may be due to variations in sources of error between laboratories, such as cytosol preparation from tissue samples, receptor assays and/or the associated protein measurements.

The relative importance of these various sources of error may be evaluated for the May 1987 trial. The same ER concentration should have been obtained for both tissue and cytosol samples in this trial since the same material, as well as homogenization buffer, was used to prepare the samples (see Materials and Methods). The ER results from tissue samples are compared to those from cytosol samples in Fig. 5. The groups within the shaded areas A and B appeared to prepare cytosols from tissue samples which yielded results similar to the cytosol samples provided by the reference laboratory, since they were grouped around the line of identity. Furthermore, it may be speculated that group A measures ER different to group B. Thus, group A was the laboratories in Fig. 3 that showed a relative change in ER measurements during the later trials from medium to high compared to the other participants. However, in addition to receptor assays, differences in the associated protein assays may also have caused a spread in ER concentration as expressed per mg protein within and between the two groups. In contrast to groups A and B, group C in Fig. 5 appeared to obtain higher ER concentration in cytosol samples as compared to tissue samples. The relatively low ER (per mg protein) in tissue samples obtained by group C may have been caused by receptor degradation during cytosol preparation of tissue samples, since error in protein measurement due to any differences in buffer composition between tissue and cytosol samples can be discounted (see Materials and Methods).

It is also important to note from Fig. 5 that none of the participants prepared cytosols from tissue samples which gave higher ER concentrations than the standard cytosol samples prepared and distributed by the reference laboratory, indicating that the reference cytosol samples were of acceptable quality. Furthermore, the tissue samples also appeared to be of acceptable quality and homogeneity since 10 out of 14 participants obtained similar results from both sets of samples. It should also be noted that no correlation between distance of transport and receptor measurements were observed, and that when tissue samples were sent to one of the participants with an interval of 3 weeks, the ER concentrations obtained were not significantly different, the mean (± S.D.) receptor levels being 48 (± 12) and 46 (± 15) fmol/mg protein respectively. It is therefore unlikely that transport and storage of tissue samples were major sources of between laboratory error.

Cytosol preparation from tissue samples may also be an important source of error for PR measurements as indicated by comparing the results for cytosol samples in the June 1986 trial with those from tissue samples in the February 1986 trial. Thus, laboratories which measured low PR in tissue samples obtained PR values comparable to the rest of the participants when assaying the reference cytosol samples as shown by the horizontal shaded area in Fig. 6.

CONCLUSIONS

The overall achievement that occurred during the AQA Programme was a reduction in between laboratory CV for both ER and PR measurements (see Figs. 1 and 2). However, the data suggest that several laboratories still have major problems with both cytosol preparation and receptor assays (see Tables 1 and 2, and Figs. 5 and 6), indicating that

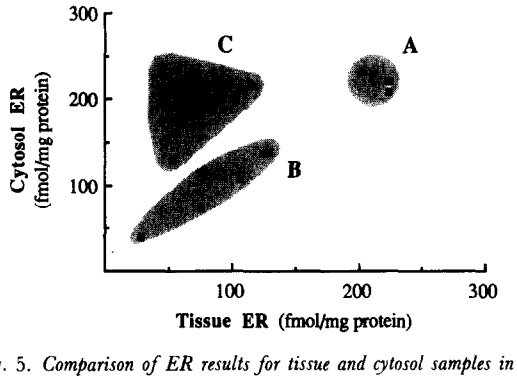

Fig. 5. Comparison of ER results for tissue and cytosol samples in the May 1987 trial. The shaded areas represent groups of laboratories (A, B and C) where the results appeared to be due to different sources of error.

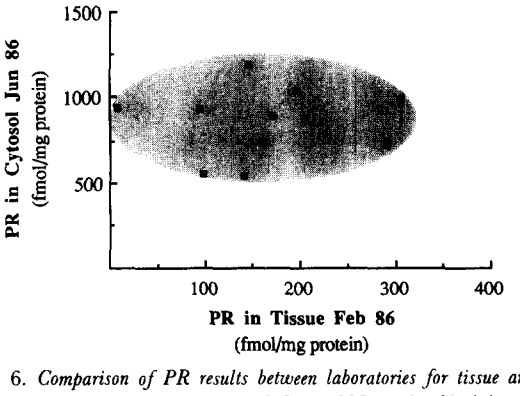

Fig. 6. Comparison of PR results between laboratories for tissue and cytosol samples in the February and June 1986 trials. Shaded area illustrates relationship between PR results for tissue and cytosol samples.

Table 2. Mean PR (fmol/mg *protein) measurements* (n = 4) *by individual laboratories in the AQA programme from* 1985 *to* 1986. *Within laboratory % CVs are given in parentheses*

Date Sample Lab I.D.	Apr 1985 Tissue	Sept 1985 Tissue	Feb 1986 Tissue	Jun 1986 Cytosol
1	79 (21)	215 (25)	113 (16)	—
2	77 (77)	500 (26)	172 (38)	884 (10)
3	198 (28)	—	197 (24)	1030 (13)
4	95 (116)	50 (97)	—	—
5*	—	—	—	—
6	140 (17)	216 (8)	142 (47)	536 (19)
7	224 (34)	220 (21)	162 (19)	736 (9)
8	247 (15)	282 (21)	96 (5)	927 (8)
9	96 (39)	335 (34)	147 (8)	1186 (4)
10	274 (15)	412 (32)	306 (68)	984 (19)
11	195 (34)	—	—	697 (18)
12	2 (17)	—	—	—
13	3 (179)	64 (28)	99 (24)	542 (3)
14	—	197 (18)	292 (11)	718 (2)
15	13 (200)	2 (76)	9 (74)	935 (21)
16	483 (18)	251 (30)	—	871 (5)
17	85 (39)	32 (29)	—	705 (1)
Mean	147 (92)†	214 (74)	158 (66)	827 (25)

*Laboratory 5 did not perform the PR assay.
†Overall % CV.

further trials are needed to monitor performance and facilitate continued evaluation of the sources of error between and within laboratories.

It was recognized from a previous trial [9] that tissue samples were essential for evaluation of the reliability of receptor measurements in breast cancer biopsies for diagnostic purposes. The results from the AQA Programme confirm that samples of tissue fragments, as prepared by the reference laboratory, were of sufficient homogeneity and stability to enable detection of major discrepancies in receptor measurements between laboratories (see Figs. 1 and 2). In fact, it was found that although a laboratory may obtain acceptable results with cytosol samples, the same may not be true for tissue samples. Thus, several laboratories under-estimated receptor concentration in tissue samples, but not in cytosol samples (see Tables 1 and 2), indicating that cytosol preparation from tissue samples was one major source of between laboratory variation (see Figs. 5 and 6). However, although our results indicate that cytosol preparation is important, it was also found that the method of protein measurements and differences in receptor assays may be the main sources of error between some of the laboratories (see Fig. 5). The reduction in between laboratory CV that was achieved during the programme is encouraging, but clearly further studies are needed so that constructive measures and recommendations can be made to improve the value of quantitative receptor measurements as a diagnostic tool for predicting response to endocrine therapy in breast cancer patients.

Acknowledgements—The authors are indebted to Greg Egan, Garvan Institute of Medical Research, Sydney, for writing the computer program which facilitated rapid distribution of the results after each trial. The excellent technical assistance provided by B. Dean, Endocrine Laboratory, Royal Melbourne Hospital, C. Gale, Waikato Hospital, Hamilton, K. Goldsmith. Flinders Medical Centre, Adelaide, H.S. Koh, The Royal Women's Hospital, Melbourne, B. Rice, Garvan Institute of Medical Research, Sydney, and E. Twaddle, King Edward Memorial Hospital for Women, Perth, was very much appreciated.

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