The use of latex beads in external quality assurance and internal quality control for routine semen analysis

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SUMMARY
The usefulness of latex beads of defined concentration was assessed as a substitute for sperm in the performance of External Quality Assurance (EQA) and Internal Quality Control (IQC) of semen analysis. Within the EQA programme, mean±SEM bias (%) was significantly reduced in 2007 compared to 2002 for both specialist (6.0%±5.4% vs. 55.0%±5.9%) and non-specialist (18.4%±5.9% vs. 90.9%±13.4%) laboratories (both p<0.0001), indicating improved accuracy over time. Within the IQC programme, the beads were used in the appraisal of two scientists, one experienced and one inexperienced, against a known standard. Beads were also used to calibrate eleven counting chambers, resulting in one old chamber being discarded due to its poor performance. The present study has shown that the use of a defined concentration of beads is an excellent adjunct to IQC and EQA programmes enabling the performance of both people and equipment to be assessed in an objective manner. Reproductive Biology 2011 11 3: 264-275.

Key words: quality control, semen analysis, latex beads

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2 Declaration: The latex beads were supplied by EQASRM for evaluation in the clinical setting. EZ is a Director of EQASRM and SJ is the Scientific Consultant.
INTRODUCTION
Assisted Reproductive Technology (ART) laboratories, including diagnostic andrology laboratories, in many countries require the mandatory adoption of a Quality Management System (QMS) which is clearly visible to users of the services provided [25]. As part of this process, accuracy and precision of any medical laboratory test must be validated through Internal Quality Control (IQC) and External Quality Assurance (EQA) programmes, and measurements of uncertainty be quantified. Australia is just one example where the process is required if payment is to be secured for recognized tests through the government’s Medicare system [17-19].

Manuals for the performance of semen analysis have been made available from the World Health Organization and these have proved valuable in standardizing practice, evolving with increased detail and complexity and culminating in the recent 5th edition [28]. Checking the performance of both equipment and personnel is covered extensively in the current edition and will no doubt be beneficial to laboratories in providing an acceptable professional standard. However, methods for quality control need not be confined to those set out in the WHO manual. The use of latex beads to assess the accuracy and precision of methods for counting sperm can complement the measures recommended in the manual. Whilst the evaluation of methods or personnel in estimating sperm concentration is limited by not knowing the true value, latex beads of a known concentration have long been recognized as a useful tool in the quality control of counting sperm [21], comparing the accuracy of different counting chambers [23, 24], and identifying sources of error associated with counting chambers [15].

The current study describes the usefulness of latex beads in the quality control of semen analysis. The beads were used in an internal quality programme of an andrology laboratory in two ways, namely 1/ checking the performance of the counting chambers and using the data to remove from service chambers that were sub-standard, and 2/ the evaluation of an inexperienced technician relative to an experienced counterpart. The beads were also employed in an EQA programme so that bias could be calculated from a known target value rather than a mean of the participants, and the value of the scheme was gauged by the change in average bias seen over a five year period.
MATERIALS AND METHODS

Beads
Latex beads with a diameter of 6.4 µm (Product # SD-6A; Sigma-Aldrich, Castle Hill, NSW 1765, Australia) were suspended at known concentrations in a colloidal fluid based on 4% modified gelatin extract (Gelofusine; B Braun Australia, Bella Vista, NSW 2153, Australia) as they have been shown previously to settle slower in medium containing protein thereby mimicking the effect of seminal plasma [15]. The beads were obtained from the supplier at certified concentrations for each lot number, and dilutions were then made to obtain the required concentration of beads. Suspensions of beads were adjusted and verified manually using an improved Neubauer haemocytometer by staff of the External Quality Assurance Schemes for Reproductive Medicine (EQASRM, PO Box 162, Northlands WA 6905, Australia) prior to distribution to the laboratories.

Dilution of beads compared to that of sperm
The suitability of latex beads suspended in Gelofusine as a surrogate for sperm in semen was tested by dilution of beads with neat Gelofusine and sperm with seminal plasma from a vasectomized man. This simple test reveals any differences in settling and flow of the two types of particles, and the behavior of the beads and sperm was then compared by correlating the actual with the expected concentration, and assessing visually the parallelism of the two dilutions.

Internal quality control
Two sets of beads were sent to a specialist laboratory. The first was a single suspension of beads at a concentration of 17×10^6/ml used to check the accuracy of the counting chambers within that laboratory. This was done 10 times for each counting chamber as follows:

a) Makler chamber (Sefi Medical Instruments, Haifa, Israel). After adequate resuspension, a 3 µl drop of the neat suspension was applied to the chamber and the number of beads counted in the whole grid. This value was divided by 10 to give the concentration in millions per ml;

b) improved Neubauer haemocytometer (various manufacturers). The bead suspension was diluted 1:5 with sperm immobilizing solution [27] and applied to both sides of the chamber. A total of at least 200
beads were counted on each side, and an average for the chamber was calculated from the two sides if they were within 5% of each other.

The mean and 95% confidence limits were calculated for the 10 replicates of each chamber, and the chamber accepted as being usable if the target value of $17 \times 10^6$/ml fell within these limits. Unacceptable chambers were removed from service.

The second lot of beads was supplied as 10 suspensions covering the range of $5 \times 10^6$/ml-$50 \times 10^6$/ml in increments of $5 \times 10^6$/ml. These beads were used in the comparison of an inexperienced technician having just completed her training with an experienced technician, and measurements made on a Makler chamber as described above. Comparisons were made initially as a correlation between the results of the two technicians, and then using a Bland Altman plot [4] modified so that the abscissa was the target value rather than the mean between the two technicians.

**External quality assurance**

The latex bead suspensions were posted to laboratories subscribing to the sperm concentration module of the Australian external quality assurance programme (EQASRM), established through the Fertility Society of Australia [14]. Distributions were made quarterly by post according to a pre-determined schedule together with other samples, and results returned to the organizer within four weeks. Bias of the results for the bead samples was calculated from the known target value of the beads and the actual result obtained by the laboratory as:

$$\text{Bias} \% = \frac{(\text{actual} - \text{target}) \times 100}{\text{target}}.$$ 

All results for samples distributed in 2007 were analyzed for the specialist laboratories in the scheme of the day (i.e. accredited by the Reproductive Technology Accreditation Committee [RTAC] of the Fertility Society of Australia [7, 8]; 129 results from 38 laboratories), and the non-specialist laboratories also participating (i.e. not accredited by RTAC but accredited by the National Association of Testing Authorities [NATA], Australia; 445 results from 118 laboratories). The mean bias shown by all laboratories for all bead samples was then compared with similar data from the distributions made five years earlier in 2002 for the specialist (111 results from 35 laboratories) and non-specialist (221 results from 68 laboratories) laboratories subscribing at that time.
Statistical analysis
Correlations and calculations of mean and 95% confidence limits were made using Excel (Microsoft Office Professional Edition, 2003). Comparisons of bias were done with the Student’s t-test in Excel and differences considered significant if p<0.05.

RESULTS
There was good agreement between the expected and observed concentrations of beads (r=0.99) and sperm (r=0.98) when diluted with Gelofusine and seminal plasma respectively. A high degree of parallelism as shown in Figure 1 confirms that the beads and sperm behave in a similar manner in their respective fluid.

The results obtained when checking the counting chambers of one specialist laboratory are shown in Table 1. Of the 11 chambers assessed, one chamber failed and was withdrawn from service. This was a very old Makler chamber that had been used frequently over many years. The assessment of a new technician after completing the training programme, by comparing performance with an experienced technician, is summarized in Figure 2. The bead concentrations obtained by the inexperienced technician gave an excellent correlation with the results of the experienced technician (fig. 2A), although the only conclusion that could be drawn was that they both performed at the same level. A more objective assessment was then made using the target values of the bead suspensions as shown in Figure 2B. A modified Bland-Altman plot confirmed that both technicians obtained results that were acceptably close to the target values of the different suspensions tested.

The kind of counting chamber used by laboratories participating in the EQA programme is shown in Table 2. The number of specialist laboratories using haemocytometers remained constant, but nearly half of these laboratories used Makler Chambers or other kinds. Whilst a large proportion of general pathology laboratories use haemocytometers, there are many that use other kinds of haematological chamber (e.g. Kova and Vetriplast chambers). The overall results of the external quality assurance scheme distributions are shown in Figure 3. There was an improvement in performance of laboratories as revealed by a significant reduction in bias from the bead target values seen between 2002 and 2007 for samples analyzed by both specialist and non-specialist laboratories (both p<0.0001).
Figure. 1. A comparison between the dilution of latex beads with Gelofusine and sperm with seminal plasma. Aliquots of sperm or bead suspensions were diluted with the respective diluent and the concentration determined using a haemocytometer. Correlations between the observed and expected values were calculated for the beads ($r=0.99$) and sperm ($r=0.98$).

Figure. 2. The two scientists, one experienced and one inexperienced, measured the bead concentration of 10 different samples to allow comparison (A) against each other by correlation, and (B) against the target bead concentration using a Bland Altman plot.
Figure 3. A comparison of the bias shown by specialist laboratories accredited by the Reproductive Technology Accreditation Committee of the Fertility Society of Australia and general pathology laboratories in measuring the concentration of beads in all samples sent out in 2002 (111 results from 35 specialist laboratories, 221 results from 68 general laboratories) and five years later in 2007 (129 results from 38 specialist laboratories, 445 results from 118 general laboratories). Values are expressed as the mean±SEM.

Table 1. The calibration of counting chambers within a single laboratory as part of routine laboratory surveillance

<table>
<thead>
<tr>
<th>Chamber</th>
<th>Bead concentration (×10⁶/ml)</th>
<th>Decision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Makler 1</td>
<td>17.00</td>
<td>16.00–18.00</td>
</tr>
<tr>
<td>Makler 2</td>
<td>18.47</td>
<td>16.73–20.21</td>
</tr>
<tr>
<td>Makler 3*</td>
<td>21.60</td>
<td>17.95–25.25</td>
</tr>
<tr>
<td>Haemocytometer 1</td>
<td>15.10</td>
<td>12.82–17.38</td>
</tr>
<tr>
<td>Haemocytometer 2</td>
<td>17.20</td>
<td>15.63–18.77</td>
</tr>
<tr>
<td>Haemocytometer 3</td>
<td>15.10</td>
<td>12.62–17.58</td>
</tr>
<tr>
<td>Haemocytometer 4</td>
<td>15.80</td>
<td>14.55–17.06</td>
</tr>
<tr>
<td>Haemocytometer 5</td>
<td>16.20</td>
<td>14.37–18.03</td>
</tr>
<tr>
<td>Haemocytometer 6</td>
<td>18.70</td>
<td>16.12–21.28</td>
</tr>
<tr>
<td>Haemocytometer 7</td>
<td>17.20</td>
<td>16.37–18.03</td>
</tr>
<tr>
<td>Haemocytometer 8</td>
<td>17.50</td>
<td>15.94–19.06</td>
</tr>
</tbody>
</table>

Beads at a target of 17×10⁶ per ml were counted ten times on each of the laboratory’s counting chambers, and the chambers accepted if the target value fell within the 95% confidence limits of the measurements; *This chamber was very old and well used, and was removed from service following this check.
**DISCUSSION**

Semen analysis is notoriously unreliable [10], with a major contributing factor being the lack of standardization of methods and reporting [12]. The provision of a reference manual by the World Health Organization has certainly been a major step in helping laboratories reduce technical variation [28], although there is often resistance to adopt these recommended protocols [22]. The use of a range of tools for monitoring the performance of personnel and equipment is clearly outlined in the current WHO manual [28] and, whilst it is commendable and extensive, it should not be seen as restricting the way one applies quality control procedures. A good example is the use of latex beads which have been known for many years to be a good substitute for sperm during an assessment of sperm counting methods [15, 21, 23, 24]. The use of beads was clearly supported in the previous edition of the WHO manual [27] but has been omitted in the current edition [28]. The present study has shown how beads can be used successfully in internal quality control and external quality assurance programmes, and would argue for the use of beads by laboratories to complement the measures outlined in the current WHO manual.

The valid use of latex beads as surrogates for sperm within quality control and assurance programmes is only possible if the beads behave in a similar fashion to sperm in semen. It would appear that the matrix in which the beads are suspended is very important, and solutions such as saline result in

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**Table 2.** The number of specialist and general pathology laboratories using various kinds of counting chamber in 2002 and 2007

<table>
<thead>
<tr>
<th>Laboratory type</th>
<th>Chamber used</th>
<th>Year</th>
<th>2002</th>
<th>2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specialist</td>
<td>Total</td>
<td>35</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haemocytometer</td>
<td>18 (51.4%)</td>
<td>20 (52.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Makler</td>
<td>15 (42.9%)</td>
<td>13 (34.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>2 (5.7%)</td>
<td>5 (13.2%)</td>
<td></td>
</tr>
<tr>
<td>General Pathology</td>
<td>Total</td>
<td>68</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haemocytometer</td>
<td>44 (64.7%)</td>
<td>58 (49.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Makler</td>
<td>3 (4.4%)</td>
<td>3 (2.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>21 (30.9%)</td>
<td>57 (48.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Specialist laboratories were those accredited by the Fertility Society of Australia through its RTAC scheme of the day.
rapid settling compared to serum or seminal plasma [15]. The beads in the present study were suspended in Gelofusine, a gelatine solution designed as a colloidal plasma volume substitute. The dilution of the beads and sperm showed parallelism (fig. 1) which implies that they behaved in a similar fashion during the handling and loading onto the haemocytometer when determining the concentration.

Identification and elimination of inter-operator error is important in minimizing variability in semen analysis [6]. In the present study, the beads were used to compare the performance of two technicians, one experienced and the other inexperienced. A simple correlation as shown in Figure 2A only allows a comparison of the inexperienced technician relative to the experienced one, the conclusion being that they operate to the same level with the possibility that undefined errors made by the experienced technician were passed on during training. The use of the beads allowed both operators to be judged against an objective standard, a method which allows independent appraisal of both technicians.

The beads have also been used in the present study to calibrate the counting chambers in a laboratory. The WHO manual only recommends that the depth of the counting chamber be checked [28] but the use of beads would seem a better reflection of sperm counting provided the errors associated with the other aspects of the analysis (e.g. pipetting) are not disproportionately large. The bead concentration was measured 10 times with each chamber, and 95% confidence limits calculated since that was more stringent than the two standard deviations recommended in the WHO manual [28], although laboratories should define their own criteria for acceptance and rejection. Chambers in the laboratory here were rejected and removed from service if the bead target value fell outside of the 95% confidence limits. Only one chamber failed the test and this was a very old Makler chamber that had been used heavily over a number of years, and had fallen out of favour when analyzing samples routinely; the beads allowed an objective assessment to be made to confirm or refute the intuition of the staff. Interestingly, the chamber was seen to overestimate the bead concentration and this phenomenon has been described previously whereby chambers deteriorate with routine wear and tear [26]. There were two main difficulties with the appraisal of the performance of the Makler chambers in the present study, namely 1/ there is no consensus about the volume of semen to be applied to the chamber (the manufacturer simply recommends a small drop of semen of undefined volume) making standardization of use between laboratories impossible, and 2/ the counting of sufficient particles in samples containing low numbers of beads (the semen is
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not diluted as with the haemocytometers and so neat semen gives no opportunity for concentrating the sample). Makler chambers have not been endorsed by the World Health Organization in the recent manual due to concerns over their performance [28]. Table 1 shows that haemocytometers are not as widely adopted as some would like, and that Maklers remain popular with specialist laboratories (presumably because of their ease of use and possible estimation of sperm concentration and motility at the one time) whilst other haematological chambers are popular with general pathology laboratories (presumably because of their familiarity when assessing blood). The German (QuaDeGA; [5]) and Spanish [1] EQA schemes also found that <50% laboratories were using haemocytometers as recommended by the WHO. Nevertheless, if laboratories claim to be using the WHO manual as their reference source (as many are now doing to satisfy mandatory accreditation) then they should not use a Makler chamber without first validating and verifying the results obtained compared to haemocytometers, and they must state that it is a deviation from the reference.

EQA is a recognized component of quality control within the andrology laboratory [28], but whilst these views are not universally held [9] there are good arguments for it [20]. A number of schemes have demonstrated that variability exists between laboratories when counting sperm [1, 5, 11, 13, 16], but the present study has described an improvement in performance over time as judged by the reduced bias. The reason for this improvement is hard to say, and while standardized training can clearly help [2], one would hope that the use of objective tools to assess one’s own performance relative to others would be a strong motivation to improve, especially if the laboratory was shown to be an outlier [3]. The value of educational activities in improving awareness, helping with standardization and generally giving support is also not to be underestimated [2], and professional bodies around the world should be encouraged to organize courses and symposia in a way that is accessible to all.

In summary, the use of latex beads provides an excellent substitute for sperm within a quality control programme for semen analysis. An objective appraisal of the laboratory performance can be made, sources of error identified, and the beads provide a simple adjunct to the quality measures promulgated within the current semen analysis manual produced by the WHO.

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