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Andrology

External quality control program for semen analysis: Spanish experience

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***Purpose*:** Results from an external quality control programme for semen analysis carried out in Spain are analysed.
***Methods*:** Quality control materials were distributed and the following seminal parameters were determined: concentration, total motility, progressive motility, rapid progressive motility, morphology and sperm vitality. The between-laboratories coefficients of variation were assessed on different types of quality control material.
***Results*:** The majority of participating laboratories utilised manual versus computer-assisted semen analysis methods. Some between-laboratories coefficients of variation ranges were: 20.8–33.8% for concentration (semen pool suspension); 13.9–19.2% for total motility (videotapes); 54.2–70.2% for sperm morphology (strict criteria using stained smears); and 9.8–41.1% for sperm vitality (stained smears). There was an inverse relation between mean percentage of sperm and coefficients of variation between laboratories for sperm motility, morphology and vitality.
***Conclusions*:** These data highlight the urgent need for improvement in the overall quality of andrology testing.

KEY WORDS: External quality control; semen analysis; sperm concentration; sperm morphology; sperm motility.

INTRODUCTION

Precise semen analysis is the most important test for determining fertility potential in the male, and the necessity for external quality control of this semen analysis has been highlighted in numerous studies (1–8) and further afield (9).

In semen analysis, there exist many possible sources of variability and thus it is necessary to control the factors that could influence results. These factors may be physiological, methodological or related to the operator's experience. The first two factors can be reduced by the standardisation of pre-analytical and analytical procedures, while the third requires technical expertise, procedural care, meticulous quality control, both internal and external and participation in internal and external proficiency testing programs.

Various editions of the WHO Guidelines (9,10) have established standardised protocols for the performance of semen analysis and related procedures. This standardisation provides the basis for the introduction of both internal and external quality control procedures. With regard to internal quality control, the guidelines establish the techniques required to maximize precision, accuracy and competence in the andrology laboratory. In addition, the desirability of developing external quality control programmes is

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stressed; results need to be transferable so that the imprecision of analytical methods in the internal laboratory can be measured, in order to compare the inaccuracy of this internal laboratory with that of others and to support the internal quality control process.

The above reasons, together with the absence of an external quality control programme in Spain, led us to establish, to develop and to evaluate a programme with these characteristics.

MATERIAL AND METHODS

Firstly, we carried out a pilot study in 1998 to evaluate the viability of this project. We mailed various control materials for each seminal parameter (with unknown values) to the different laboratories participating, in order to choose the most suitable material for further trials. Proficiency testing programmes were developed for the determination of sperm count, sperm motility, sperm morphology and sperm vitality. In the following year, an official programme started, with two trials per year from 1999 to 2002. The results from each laboratory participant were compared with the average obtained from all the laboratories participating in this trial, in which the laboratories applied their normal methods.

The following quality control materials were used in the pilot experiment to assess the between-laboratories variation of sperm concentration: frozen straws (0.25 ml) kept at −196°C with added cryoprotectant, aliquots of formalin (1%) semen suspension and latex beads (3 μ diameter) (SIGMA-Aldrich, Spain) suspension. These were suspended in phosphate buffered saline medium supplemented (SIGMA-Aldrich, Spain) with bovine serum albumin (SIGMA-Aldrich, Spain) (5%) and aliquoted (0.2 ml) in Eppendorf tubes prior to their distribution. The participating laboratories were instructed to remove the specimens from the refrigerator (4°C), warm to room temperature, shake until completely in suspension and count according to the laboratory's usual method. Results were recorded as $\times 10^6$ spermatozoa or beads/ml. The laboratories were required to indicate the type of counting chamber used and method (manual or CASA).

For the study of between-laboratories variation of sperm motility, we mailed videotapes (VHS) and frozen semen in straws. All straws for the motility assessment were to be thawed for 10 min at 37°C before the analysis, and reports were to be made as the percentage of motile spermatozoa (grades a + b + c), progressive motility (grades a + b) and rapid progressive motility (grade a).

For sperm morphology, frozen straws, 50 images of sperm on videotape, unstained and stained smears were mailed. The unstained smears were fixed with methanol and each laboratory was instructed to stain them by their usual method. The rapid panoptic method (Diff-Quik, Dade Diagnosis, EE.UU.) was used for the stained smears mailed. The laboratories were instructed to perform analysis of the smears by their usual method. Results were to be reported as the percentage of normal forms. Eosin Y—Nigrosin (SIGMA-Aldrich, Spain) smears to assess sperm vitality were mailed. The laboratories were instructed to record the percentage of live sperm.

The quality control material mailed within eight trials of official programme were: two aliquots of formalin sperm suspension for sperm concentration, at least two samples on videotaping for motility, two unstained and two stained semen smears for morphology and two Eosin Y—Nigrosin semen smears for vitality.

The samples used were obtained from donor candidates, all of whom previously gave their informed consent for their ejaculates to be used in the investigation. Serum studies were performed for human immunodeficiency virus, hepatitis B, hepatitis C and syphilis. All resulted negative.

Each laboratory was assigned a secret code for data processing. Prior to the distribution of the quality control material, we randomly obtained four samples and analysed them in strict accordance with the WHO (9,10) guidelines for routine semen analysis. Results were returned to participants as a report indicating the dates remitted, the number of laboratories that used the same method, the mean, standard deviation (SD), coefficient of variation (CV) and standard deviation index (SDI). The latter value indicates the number of standard deviations that a remitted value varies from the mean found for that method.

After the mean and standard deviation were calculated, the results lying outside the mean ± 3 SD interval were excluded, and mean and SD were recalculated. The correlation between the between-laboratories coefficient of variation and the mean of a given seminal parameter was recorded by the simple linear Pearson coefficient.

Table I. Techniques Used and Participation Evolution

	1998	1/1999	2/1999	1/2000	2/2000	1/2001	2/2001	1/2002	2/2002
No. of participants	18	32	28	30	29	40	37	40	40
Concentration									
Neubauer haemocytometer	0 (0%)	7 (22.6%)	5 (17.9%)	10 (33.3%)	10 (34.5%)	15 (37.5%)	16 (43.2%)	11 (28.2%)	13 (32.5%)
Makler	16 (100%)	24 (77.4%)	23 (82.1%)	20 (66.7%)	17 (58.6%)	24 (60%)	19 (51.4%)	28 (71.8%)	27 (67.5%)
Automated	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (6.9%)	1 (2.5%)	2 (5.4%)	0 (0%)	0 (0%)
Motility									
Manual	15 (93.7%)	30 (100%)	27 (96.4%)	27 (90%)	27 (96.4%)	39 (97.5%)	36 (97.3%)	37 (97.4%)	39 (97.5%)
Automated	1 (%)	0 (0%)	1 (3.6%)	3 (10%)	1 (3.6%)	1 (2.5%)	1 (2.7%)	1 (2.6%)	1 (2.5%)
Morphology									
Kruger/WHO-99	7 (46.7%)	14 (45.2%)	11 (39.3%)	20 (66.7%)	24 (82.8%)	32 (80%)	32 (86.5%)	35 (87.5%)	35 (87.5%)
WHO-92	8 (53.3%)	17 (54.8%)	17 (60.7%)	10 (33.3%)	5 (17.2%)	8 (20%)	5 (13.5%)	5 (12.5%)	5 (12.5%)
Vitality									
WHO-92/WHO-99		28	26	29	28	38	35	38	39

RESULTS

Participation in the external control quality programme increased during successive trials (from 18 laboratories in the pilot study to 40 in the last trial) (Table I). The main techniques used to determine concentrations were the Makler counting chamber and the improved Neubauer method, while very little automated analysis was performed. In motility analysis, manual methods were normally preferred to automated systems. In both cases, automated dates were eliminated from the calculations. Finally, the use of WHO-92 criteria to determine normal sperm morphology decreased in the course of the study, whereas strict WHO-99 criteria were increasingly applied (Table I).

No significant correlations were observed between concentration means using semen pool and between-laboratories CV ($r = 0.13$), or between the means of normal forms by strict WHO-99 criteria using stained smears and between-laboratories CV ($r = -0.29$). However, we did observe a significant relationship between the means of total and progressive motility using videotapes ($r = -0.80$ and $r = -0.79$, respectively), the mean of normal forms by WHO-92 criteria using stained smears ($r = -0.55$), and the mean of live forms ($r = -0.86$) and between-laboratories CV (Fig. 1).

Table II shows the between-laboratories CV obtained in the first trial for each quality control material. On the basis of these results, the ease of preparation and the cost, we decided the quality control materials to be mailed as part of the official programme in subsequent years.

The between-laboratories coefficients of variation ranges for seminal parameters were 20.8–33.8% for concentration by semen pool suspension; 13.9–19.2% for total motility, 17.3–27.0% for progressive motility and 48.1–70.4% for rapid progressive motility using videotapes; 33.5–75.0% for sperm morphology following WHO-92 criteria using unstained smears and 30.3–81.1% using stained smears, and 55.7–86.7% following strict criteria using unstained smears and 54.2–70.2% using stained smears; and 9.8–41.1% for sperm vitality using stained smears. We did not observe any great changes in the temporal evolution of between-laboratories CV for concentration and percentage of total and progressive motility (Table III; Fig. 2). However, we did observe an erratic temporal evolution of the between-laboratories CV for rapid progressive motility (Table III; Fig. 2), live and normal sperm, irrespective of the criteria used or types of smears analysed (stained or unstained) (Table IV; Fig. 3).

DISCUSSION

Obtaining reliable laboratory data for the diagnosis and treatment of subfertile patients, in conjunction with the great interest that semen analysis has aroused after reports of decreasing seminal quality in developed countries, has led to the development of external quality control programmes (1,2,6,11–14). Participation in our external quality programme had extended to 40 laboratories by the last two trials, a participation superior to that of similar experiences (1,2) although less than that of national programmes, such as those carried out in the UK (4) and in the US (6).

The WHO manual (1999) recommends the use of Neubauer haemocytometers and the fact that we

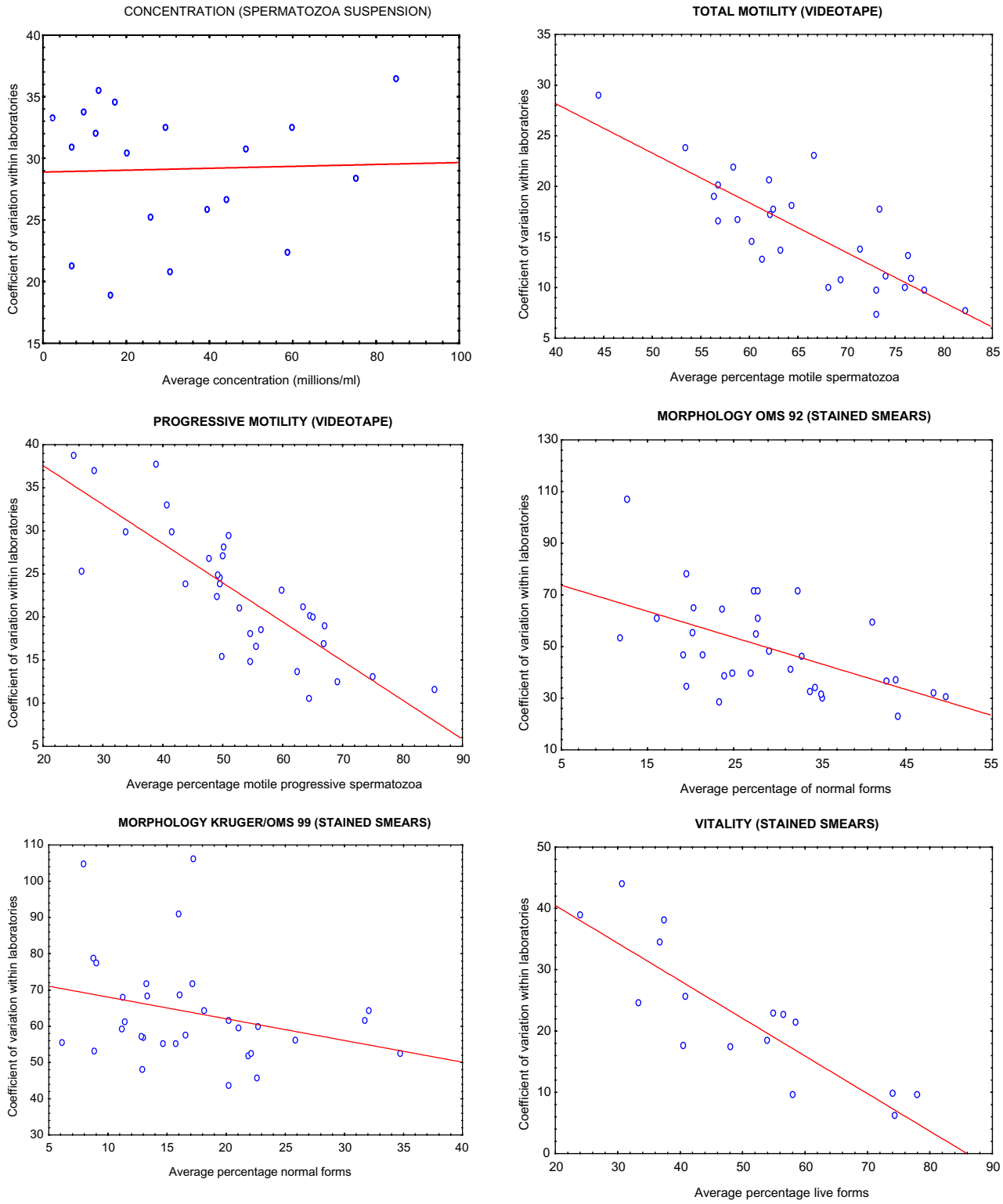

Fig. 1. Correlation between concentration or percentage/motility, morphology and vitality, mean and between-laboratories CV for each seminal parameter.

Table II. Results Obtained for the First Trial Each Material

	Labs number	Mean	CV
Concentration ($\times 10^6$ spermatozoa/ml)			
Cryopreserved semen	16	59.3	39.5
Semen pool suspension[a]	16	30.6	28.9
Latex particles	15	339.4	29.8
Videotape	29	88.5	25.7
Motility a + b + c (% motile forms)			
Videotape[a]	27	69.6	13.9
Motility a + b (% motile forms)			
Cryopreserved semen	13	20.2	34.5
Videotape[a]	30	55.5	23.2
Motility a (% motile forms)			
Cryopreserved semen	13	9.4	51.2
Videotape[a]	30	31.6	59.0
Morphology (WHO-92) (% normal forms)			
Cryopreserved semen	7	31.9	48.7
Videotape	16	23.9	28.4
Unstained smears[a]	16	23.4	75.0
Stained smears[a]	8	20.5	51.0
Morphology (Strict/WHO-99) (% normal forms)			
Cryopreserved semen	6	15.4	43.9
Videotape	14	19.0	42.7
Unstained smears[a]	13	12.1	59.3
Stained smears[a]	7	9.6	56.3
Vitality (% live forms)			
Stained smears[a]	28	34.0	41.1

[a]Material used in posterior trials.

observed a progressive increase in their use means that WHO recommendations are slowly being incorporated into Spanish centres. On the other hand, Baker *et al.* (15) and Keel *et al.* (6) reported a greater use of Neubauer haemocytometers. In contrast, we found little use of automated systems in Spain (0.5%), whereas Keel *et al.* (6) reported that 15% of laboratories use these systems.

Our results showed a higher between-laboratories CV for sperm concentration using frozen straw than semen suspension. This finding coincides with those of other studies (4,16,17) that have reported a lack of consistency between cryopreserved semen aliquots when these are used for internal or external quality control programmes. Thus, between the two types of samples suggested by the 1999 WHO manual for quality control concentration (cryopreserved semen and semen preserved with formalin) we chose the fixed semen pool suspension, because of its simple preparation, low cost and lower between-laboratories coefficient of variation.

The lack of relation between the concentration mean and the between-laboratories CV is contradicted by Neuwinger *et al.* (2), but these authors found an inverse relation when they analysed samples with a concentration range that involved lower values (4.6×10^5 spermatozoa/ml) than the minimum concentration value we achieved (2.1×10^6 spermatozoa/ml). In a more recent study, Auger *et al.* (7) reported that the variations found in semen analysis are higher when high concentration levels are analysed. They suggested that different count chambers, different dilutions applied for high concentrations and the use of different pipettes could be the reason for this large variation. However, as the highest quantity we analysed was 84.5 millions/ml in comparison with the 20% of samples that had a higher concentration in Auger *et al.* (7), it is reasonable to consider that the dilution factors had less influence in our coefficients of variation.

In motility determination, our coefficients of variation and those reported by Neuwinger *et al.* (2)

Table III. Evolution of the Between-Laboratories CV

	1998	1/1999	2/1999	1/2000	2/2000	1/2001	2/2001	1/2002	2/2002
Concentration (semen pool suspension)									
N (Labs number)	16	31	27	29	26	39	32	39	39
Mean ($\times 10^6$ ml)	30.6	52.3	29.2	46.2	37.4	25.5	18.1	32.6	12.8
CV (%)	28.9	33.5	32.4	31.5	20.8	28.9	26.9	25.6	33.8
Motility a + b + c (videotape)									
N (Labs number)			27		27	38	37	40	40
Mean (% motile forms)			69.6		64.6	66.7	62.4	64.0	60.9
CV (%)			13.9		18.8	12.2	19.2	14.5	18.5
Motility a + b (videotape)									
N (Labs number)		30	27	26	27	39	37	39	40
Mean (% motile forms)		55.5	59.9	45.5	52.3	54.6	50.7	51.5	48.0
CV (%)		23.2	17.3	22.8	25.7	17.4	27.0	24.7	25.1
Motility a (videotape)									
N (Labs number)			27	26	27	39	37	39	40
Mean (% motile forms)			31.6	20.7	25.8	24.2	23.5	18.2	24.9
CV (%)			59.0	70.4	56.8	57.0	59.6	69.1	48.1

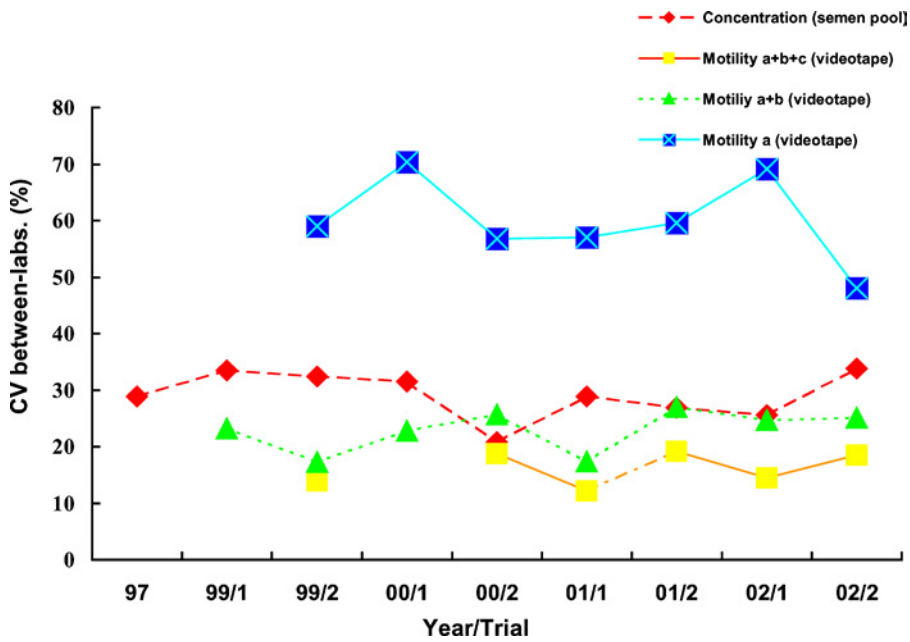

Fig. 2. Temporal evolution of between-laboratories CV (concentration and motility: grades a + b+ c, a + b, a).

show a high between-laboratories and between-technicians variability when cryopreserved semen is used, in relation to videotape. The high cost and the inconvenience of cryopreserving and mailing frozen semen, in addition to the high coefficients of variation between laboratories observed both by us and by the above mentioned authors, led us to reject this option. Nevertheless, the experience of videotape analysis is obviously different from that of microscope observation, and the results obtained are very imprecise, according to the 1999 WHO manual. To resolve this question and to avoid the bias involved in the use of monitors in external quality control, and that of microscopy in the daily routine, Kvist and Björdahl

Table IV. Evolution of the Between-Laboratories CV

	1998	1/1999	2/1999	1/2000	2/2000	1/2001	2/2001	1/2002	2/2002
Morphology (WHO-92) (unstained smears)									
N (Labs number)		16	15	7	5	8	5	5	5
Mean (% normal forms)		23.4	23.0	37.6	20.4	35.4	35.8	34.1	26.5
CV (%)		75.0	43.6	54.3	50.7	39.0	49.1	33.5	35.7
Morphology (WHO-92) (stained smears)									
N (Labs number)	8	17	16	10	5	8	5	5	5
Mean (% normal forms)	20.5	26.6	23.7	44.6	20.1	32.2	33.4	29.1	23.7
CV (%)	51.0	51.0	53.2	46.2	81.1	44.3	35.7	30.3	61.7
Morphology (Kruger/WHO-99) (unstained smears)									
N (Labs number)		13	11	20	24	32	29	35	35
Mean (% normal forms)		12.1	11.8	16.5	10.6	25.9	26.5	20.8	23.2
CV (%)		59.3	66.4	55.7	86.7	79.4	62.1	55.8	63.5
Morphology (Strict/WHO-99) (stained smears)									
N (Labs number)	7	14	11	20	24	31	30	35	35
Mean (% normal forms)	9.6	12.4	12.1	17.9	14.5	24.4	19.1	19.3	19.3
CV (%)	56.3	61.0	58.1	58.8	70.2	66.8	54.2	55.2	75.5
Vitality (stained smears)									
N (Labs number)		28	26	29	28	38	35	38	39
Mean (% live forms)		34.0	34.9	44.2	32.3	65.9	65.8	64.5	57.4
CV (%)		41.1	29.7	17.7	32.3	9.8	14.2	15.0	22.1

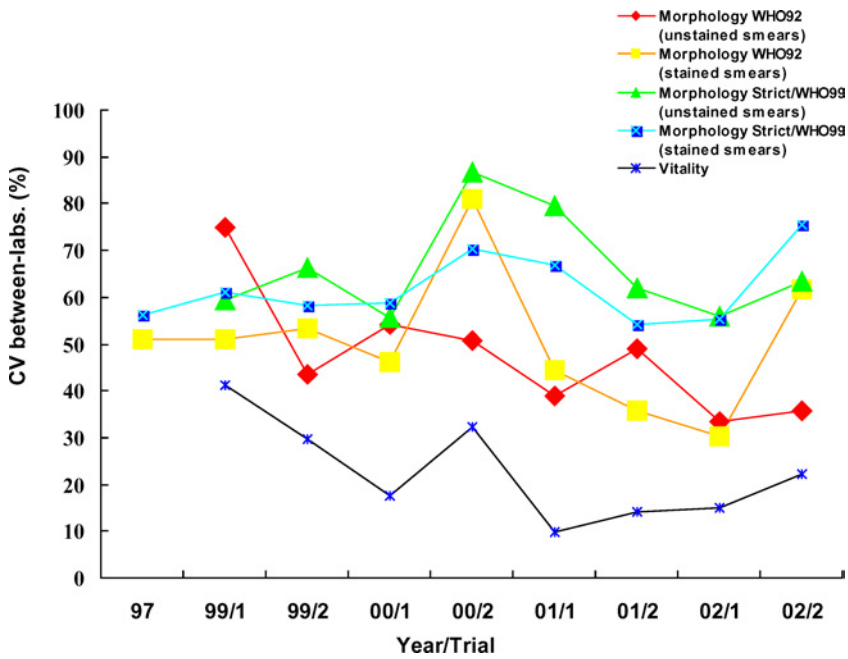

Fig. 3. Temporal evolution of between-laboratories CV (morphology: WHO-92 and Kruger/ WHO-99 criteria in unstained and stainedsmears; and vitality).

(18) suggested it is advisable use monitors in routine sperm motility evaluation.

The inverse relation we observed between the percentage of motile spermatozoa and the between-laboratories variation is similar to that reported by Neuwinger *et al.* (2) in the mailing of cryopreserved semen, and by Cooper *et al.* (4), who used videotapes. This fact could be due to the greater difficulty in evaluating an adequate number of spermatozoa in poor quality samples, due to an increase in analytical imprecision.

Although Keel *et al.* (6) and Davis and Gravance (19) suggested that a large part of the between-laboratories variability in evaluating sperm morphology is due to spread and stain techniques, we believe that differences in the application of morphologic criteria must be the main factor responsible for variations between laboratories, as is suggested by the similar between-laboratories CV obtained in the evaluation of unstained and stained smears, and which is in agreement with other authors (20). We believe the use of micrometers, as recommended in the 1999 WHO guide, could help to reduce these differences.

The inverse relation observed between the mean percentage of normal forms and the between-laboratories CV could explain the higher variation between laboratories obtained within laboratories using strict WHO-99 criteria. This coincides with the results of other authors who have studied within-laboratory variability (16) or between-laboratories variability (6) in sperm morphology. Thus, Coetzee *et al.* (14) suggested a higher number of spermatozoa are analysed when strict morphologic criteria are applied.

For the vitality analysis, we chose stained smears to avoid variations due to preparation and the staining of smears, and because of their low cost and simple use. The inverse relation between the percentage of live forms and the between-laboratories coefficient of variation is similar to that reported by Walker (12). Our coefficients of variation between laboratories are similar to those found by Keel *et al.* (6) who used stained smears (by eosin–nigrosin stain) as the control material, and very different from those reported by Walker (12) using cryopreserved semen (range 42–90%).

If we compare our results with those of other quality control schemes and take into account the previously reported acceptable degree of imprecision for all seminal parameters (21), we see that the between-laboratories CV obtained in the Spanish programme for concentrations are similar to those reported by Matson (2) in the UK and by Neuwinger *et al.* (1),

and are clearly lower than those obtained by Keel *et al.* (6) in the USA. If we compare this coefficient of variation with the minimum desirable imprecision, we see that its value approaches that obtained for this seminal parameter (≤20.1%) (21). For motility, our between-laboratories coefficient of variation is similar to that found by Neuwinger *et al.* (1) and very close to the level of minimum desirable imprecision (≤13.8) (21).

The between-laboratories variation for sperm morphology found in the present study is slightly higher than that observed by other authors (2,6), and clearly worse than that obtained by Neuwinger *et al.* (1). This discrepancy could be due to the fact that the percentage of normal forms obtained using WHO-92 or strict WHO-99 criteria in our programme is lower than the percentage obtained using WHO-87 criteria. These criteria were used by Neuwinger *et al.* (1), and as described above, there is an inverse relation between the mean percentage of normal forms and the between-laboratories CV. All the external quality control programmes studied for sperm morphology produce results far from the minimum desirable degree of imprecision (≤14.7) (21).

In the present study, the between-laboratories CV remained almost constant in time for concentration, total and progressive motility. However, it varied, although with no clear cut tendency, for rapid progressive motility and morphology. These discrepancies could be due to the lack of standardisation of the method and of the assessment criteria used by the participating laboratories (4) or to the lack of standardisation in the quality control material, as shown recently by Cooper *et al.* (8). These authors showed an agreement between the results of three external quality control schemes for concentration, total and progressive motility, and disagreement between those for rapid progressive motility and morphology.

In conclusion, we have shown the viability of a national external quality control programme that involves sperm concentration analysis using semen pool suspension, motility using videotapes, and sperm morphology and vitality by spread semen smears. The temporal evolution of between-laboratories coefficients of variation is high, which suggest that in order to reduce these coefficients of variation it is necessary to implement another measures beyond the consolidation and standardisation of external quality control programmes, such as the creation of follow-up training courses and the standardisation of techniques (8).

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