Running title:

Comparison of automated and manual AMH assays.

Title:

Evaluation of two new automated Anti-Müllerian hormone assays relative to the two current clinical assays.

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Capsule:

The new automated AMH assays provide values that are substantially lower than existing AMH ELISAs. Assay specific interpretation is necessary with international standardization urgently required.

ABSTRACT

Objective: To compare anti-Müllerian hormone (AMH) assay performance characteristics from the new automated Roche and Beckman Coulter assays with the existing Beckman Coulter Gen II and Ansh Labs assays.

Design: Prospective assay evaluation.

Setting: An university affiliated clinical chemistry laboratory.

Patient(s): Randomly sampled patients (n=83) referred for serum AMH at Invicta Laboratories prior to commencement of IVF cycle between September and October 2014. Laboratory and clinical investigators were blind to biochemical and outcomes respectively.

Interventions: None.

Main Outcome Measure(s): Serum AMH concentration.

Results(s): Intra-assay coefficients of variation were low; Ansh⩽9.0%, Gen II⩽5.8%, Access⩽10.7%and Elecsys⩽2.8%. The Passing–Bablok regression equations (in pmol/l) was y (Access) =0.128+(0.781×Gen II) and y (Access) = 0.302+(0.742xAnsh). For y (Elecys) = 0.087+(0.729xGen II) and y (Elecys) = 0.253+(0.688xAnsh). For y (Elecys) = 0.943-(0.037xAccess). All four assays AMH exhibited moderate positive correlation with AFC (r 0.62-0.64), number of cumulus oocyte complexes (r=0.60-0.64) and MII oocytes (r=0.48-0.50). Prediction of pregnancy as determined by area under the receiver operator characteristic curve was uniformly low for all assays (AUROC=0.62-0.63).

Conclusions: The novel automated assays exhibit strong concordance in calibration but derived values are substantially lower than obtained from preexisting assays with assay specific interpretation required for routine clinical use. These results highlight the need for an international AMH standard.

Key Words:

Anti-Müllerian hormone, assay, antral follicle count,

Introduction

Anti-Müllerian hormone (AMH) has rapidly established countenance as the ovarian biomarker of choice in reproductive medicine(1-4). With recognition of its clinical utility beyond simple ovarian response prediction(3, 5, 6), there has rapid development of several AMH assays(1). At present two ELISA AMH assays dominate the clinical arena; the modified Beckman Coulter Generation II assay and the Ansh Labs AMH assay(7-9). These ELISA assays are inevitably limited by their technical characteristics that limit their up scaling for routine clinical chemistry laboratories. Furthermore the Beckman Coulter Generation II assay exhibited complement interference in fresh samples requiring a modified protocol to be developed, introducing a further step that would complicate robotic instrumentation(10, 11).

Given the potential wide spread application of AMH measurement in several clinical spheres an accurate, robust, and automated assay was urgently required(12). Two such assays have now been developed and released by Beckman Coulter and by Roche Diagnostics on their Access and Elecsys platforms respectively(13). Although these two companies are intrinsically linked due to the use of identical antibodies in their respective automated AMH assays, assay development differed(13). Consequently Roche noted that their assay provided values which were substantially lower than the two pre-existing ELISA assays(13). In contrast documentation from Beckman Coulter suggests equivalent values of the Access AMH assay to those reported by the AMH Gen II ELISA. To date we are unaware of any external validation of the performance of these two new automated AMH assays (Beckman Coulter Access AMH assay and the Roche Diagnostics Elecsys AMH assay) relative each other and to the two current clinical standard assays (Beckman Coulter Generation II ELISA and the Ansh Labs ultrasensitive AMH/MIS ELISA).The importance of such post-marketing external validation has previously been highlighted by the release of field safety notices by Beckman

Coulter, when laboratories observed discordance between anticipated and actual results(8, 14, 15). To address this urgent need for external validation we have used a pragmatic design and compared the performance of all four AMH assays utilizing patients referred for assisted conception.

Material and methods

Serum AMH was measured in 83 randomly selected women undergoing a standard IVF cycle between September 2014 and October 2014(16). All samples were collected on cycle day 2-5, frozen at -80°C with AMH analysis performed on the same day for all four assays in accordance with manufacturers protocols after a single freeze thaw. The AMH assays used were: the Beckman Coulter modified Generation II AMH assay, Ansh Labs ultrasensitive AMH assay, Roche Diagnostics Elecsys AMH assay and Beckman Coulter Access AMH assay. For all assay systems we have presented results in ng/ml. Serum samples with results above the upper measuring range for each method were diluted automatically (using the reagents supplied by the manufacturers of the assays) and analyzed again. Manufacturer reported assay characteristics, measuring ranges, analytical sensitivity and detection limits are provided in Supplemental Material Table 1.

In the first stage of the study, the precision of the AMH tests was evaluated for two different control serum as supplied by the manufacturers (Controls 1 and 2 for Beckman Coulter Gen II, Controls 1 and 2 for Ansh Labs, PreciControl AMH 1 and 2 for Roche Elecsys and Controls 1, 2 and 3 for Beckman Coulter Access) in accordance with the Clinical and Laboratory Standards Institute protocol (CLSI). In the assessment of the accuracy of the results, we used the degree of agreement between the average value obtained from the series of control tests, and the predicted value for a particular control material level declared by the manufacturer(17).

Statistical analyses were performed using Stata version 13 and MedCalc 11.6.1.0. AMH values were log transformed to approximate normal distributions. Assay type was tested by generalized linear regression or paired t-test. Passing-Bablok regression equations were used to estimate the relationship between the results obtained with different analyses. Bland-

Altman plots were used to compare tests graphically to assess bias and check whether the variability in measures was homoscedastic. Correlations among the number of antral follicles (AFC), number of acquired cumuluses, MII, biochemical pregnancy and clinical pregnancy were evaluated by the Spearman rank correlation test. Receiver Operating Characteristic (ROC) analysis was performed to obtain the sensitivity, specificity and the cut-off value for each test. A 95% confidence interval was set as being statistically significant in all tests.

Results

Results of precision and accuracy for each AMH assay are shown in Table I. The obtained coefficients of variation (CV) for compared sets of analytical and evaluated AMH values were satisfactory. For each assay, the analyzed CV value was less than 11%. For both automated methods compared, correctness was satisfactory - in all of the analyzed cases the load did not exceed the value of 11%.

Comparative analysis of the four AMH assays was performed on a total of 83 patients with baseline and IVF outcome characteristics provided in Supplemental material Table 2. In brief women ranged in age from 24-44 years, 1-35 cumulus oocyte complexes were retrieved and 40/83 (48.2%) achieved a clinical pregnancy. Figure 1 and Supplemental Table 3 demonstrates that there was no overall difference in distribution of AMH values between the four assays ($p=0.07$). However, although the AnshLabs/Beckman Coulter Gen II values were broadly comparable ($p=0.87$), direct comparison of the other individual assay pairs demonstrated significant differences; AnshLabs/Roche Elecsys ($p<0.0001$), AnshLabs/Beckman Coulter Access ($p<0.001$), Beckman Coulter Gen II/RocheElecsys ($p<0.0001$), Beckman Coulter Gen II/Beckman Coulter Access ($p<0.0001$) and Roche Elecsys/Beckman Coulter Access ($p<0.001$).

Figure 2 presents the Passing-Bablok regressions and Bland-Altman plots comparing pairs of assays across the range of AMH values. The Passing-Bablok plots, which depict the linear regression line and 95% confidence interval (CI) relative to a slope of 1, show that there was a linear relationship between each combination of the four assays, however there was marked disparity when comparing some of the assays. In particular the new automated Beckman Coulter Access and Roche Elecsys assays gave considerably lower values than the Beckman Coulter Gen II assay and the Ansh Labs assay. Supplemental Table 2 provides the regression equations and 95% confidence intervals for the slope and intercept. Bland-Altman plots present the disparity in AMH values relative to the mean of the two assays to help determine systematic error and whether it is uniform across the range of results. The Bland-Altman plots show that as mean AMH levels increase, the differences between the two values become larger, with the exception of the comparison of the Beckman Coulter Access and Roche Elecsys assays where the relationship was constant across the range of AMH values.

In addition to these assay performance characteristics we examined the association of assay specific AMH values with the alternative ovarian biomarker antral follicle count (AFC) and clinicaloutcomes. For all four assays AMH exhibited moderate positive correlation with AFC, number of cumulus oocyte complexes and MII oocytes (Table 2). With respect to AMH prediction of biochemical and clinical pregnancy the area under the ROC curves were uniformly low (Supplemental Figure 1).

Discussion

Recognition of the clinical utility of AMH has led to its rapid adoption by many assisted conception clinical laboratories. Despite consistent evidence that AMH can predict ovarian response(16, 17), individual clinicians have had their confidence shaken due to issues with sample handling, complement interference, non-standardized reporting and lack of reproducibility(1, 18). Herein we report that the new automated assays from Beckman Coulter and Roche Diagnostics assay exhibit appropriate technical characteristics, but the lack of universal calibration means that these values are quite different from the current manual assays. Furthermore despite package inserts suggesting similar calibration for the Beckman Coulter modified Gen II and their new Access assay, we identify that the new automated assay from this manufacturer derives values that are approximately 22% lower than anticipated.

In accordance with previous reports we confirm that all four assays exhibit moderate correlations with antral follicle count and ovarian response as defined by both number of MII oocytes and total oocyte number (4, 19). This confirms that for ovarian response prediction all four assays could be potentially used, with the other technical aspects of low coefficients of variation and reproducibility driving selection. In keeping with the published literature(20), all four assays exhibited weak correlations with biochemical and clinical pregnancy. We were unable to examine the association with live birth, but we would anticipate that to be equally weak based on the strength of association with clinical pregnancy and previous meta-analyses(5, 21).

The discordance between the values obtained by the two Beckman Coulter assays is a further example of post-marketing surveillance identifying potential issues with assay performance. In the package insert of the Access assay the passing-Bablok regression is Access = (0.09 +

0.95 x Gen II), suggesting approximately similar values should be obtained between the modified Gen II assay and the new Access assay in contrast to the 22% reduction that we observe in the current study. In support of our current findings is that the Access and Elecsys assay return similar values and the reduction that we observe in the Elecsys assay values relative to Gen II and Ansh Labs are in accordance with the original manufacturers description of the assay(13). The underlying cause for this discordance is unclear but the systematic differences would suggest a difference in calibration rather than assay pharmacokinetics or linearity. Elecsys assay standardization was accomplished via sample value transfer from the Beckman Coulter AMH Gen II assay, unmodified version using an aged serum panel. However the limitations of this approach were acknowledged by Roche(13), as it does not overcome the high degree of between-laboratory variability of the manual AMH assay, it does not exclude residual complement activity interference in the aged reference serum panel and that the modified AMH Gen II assay methodology delivers increased AMH concentrations if compared to the unmodified assay or the Elecsys AMH. At present the method for Access assay standardization methodology has yet to be reported.

Currently there is no agreed international standard for AMH. This is increasingly important as routine uptake is accelerating and assays are readily available from several manufacturers, with additional manufacturers developing assays. To date we have used regression equations to facilitate pooling of AMH data derived from different sources(22). Standardization of reported values would facilitate development of general reference ranges to identify individuals or population subgroups, for example, women at risk of ovarian hyperstimulation syndrome(17, 23). The overall cost of diagnostic testing would be reduced, by negating the need for repeat testing in a clinician/assay service provider specific manner. Patient safety would also be enhanced as large differences in assay calibration may lead to misinterpretation of clinical course when different assays are used or clinicians are unaware of assay

differences. Given these issues, a human recombinant AMH international standard needs to be urgently developed and adopted.

Strengths and limitations of the study require consideration. This study represents a pragmatic comparison of AMH measurement methods using an unselected group of patients referred for clinical AMH measurement prior to undertaking assisted conception. In our center all patients routinely have AMH measured in advance of ovarian stimulation making our results generalizable to the assisted conception population. We had data on important clinical outcomes, including number of cumulus oocyte complexes, mature oocytes retrieved and clinical pregnancies. That we observe equivalent correlations and predictive capacity similar to those previously reported from much larger series gives confidence in our findings. As we suggest, recent clinical problems with the Beckman Coulter Gen II assay suggest the need for tighter regulation of this increasingly important biochemistry assay using international standards and immunoassays that perform consistently in the long term. Plans for a National External Quality Assessment Service scheme in the UK are being developed to achieve this. Lot-to-lot variation could not be assessed, and we could not run standards from the different assay manufacturers on the others platform due to species specificity of the antibodies.

In conclusion we demonstrate that all four assays can be utilized clinically for ovarian response prediction with greatest precision exhibited by the Roche automated AMH assay. However, the values generated by these assays can be markedly different and assay specific interpretation is required.

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Table 1. The results of the precision and accuracy of AMH assays (according to Immunotech: CV admissible–14,2%, B admissible–20%).

	Control sample	LOT	Nominal value [ng/ml]	Range	Average [ng/ml]	SD	CV[%]	B[%]
AnshLabs AMH/MIS ELISA	Control 1	111813	1.50	1.11-1.89	1.46	0.13	9.00	-2.5
	Control 2		4.40	3.50-5.30	4.26	0.38	8.80	-3.1
Beckman Coulter AMH Gen II ELISA	Control 1	334490	3.00	2.40-3.60	2.88	0.17	5.82	-4.0
	Control 2		8.80	7.00-10.60	8.17	0.16	1.95	-7.2
Roche Elecsys AMH	PreciControl 1	177753	0.99	0.78-1.20	0.98	0.03	2.80	-0.9
	PreciControl 2	177754	5.41	4.27-6.55	5.34	0.11	2.00	-1.3
Beckman Coulter Access AMH assay	Control 1	489202	0.86	0.584-1.13	0.86	0.09	10.76	-0.2
	Control 2		4.39	3.41-5.38	4.39	0.32	7.40	0.0
	Control 3		13.17	9.11-17.22	13.16	1.35	10.25	0.0

AMH – Anti-Müllerian Hormone, LOT – batch Lot number, SD – standard deviation, CV - coefficient of variation, B- bias

Table 2. The correlation between assay specific AMH levels and antral follicle count, number of cumulus oocyte complexesand number of MII oocytes.

	ELISA assays		Automated assays	
	Ansh Labs AMH/MIS ELISA	Beckman Coulter AMH Gen II ELISA	Roche Elecsys AMH	Beckman Coulter AccessAMH assay
AFC	0.620	0.619	0.636	0.628
Cumulus oocyte complexes	0.605	0.616	0.644	0.615
MII oocytes	0.477	0.501	0.502	0.486

All correlation coefficients $p<0.0001$

Figure Legends

Figure 1: Distribution of values measured by the four AMH assays.

Individual assay values with median values and interquartile range indicated.

Figure 2: Passing-Bablok regression (A-F) (solid line = regression curve and 95% confidence interval (CI) relative to a slope of 1) and Bland-Altman graphs (G-L) (solid line = mean difference; dotted lines = 95% CI) were obtained with the four assays. Individual Passing-Bablok regression equations are provided in Supplemental Table 4.

Supplemental Figure 1: Receiver operating characteristic curves and values (95%CI) for prediction of (A) biochemical pregnancy and (B) clinical pregnancy.

Supplemental materials:

Supplemental Table 1: Anti-Müllerian hormone (AMH) assays characteristics

Competitor Assays	**Beckman Coulter AMH Gen II**[1, 2]	**AnshLabs Ultrasensitive**[3]	**Roche Assay Elecsys® AMH**[4]	**Beckman Coulter Access 2 IA AMH**[5]
Assay type	Manual	Manual	Automated	Automated
Imprecision	< 8%	< 6%	1.8-2.0%	2.87-4.34 %
Sample type	serum, plasma	serum, plasma	serum, Li-heparin plasma	Serum, Li-heparin plasma
Minimum sample volume	20 µl	50 µl	50 µl	20 µl
Incubation time	< 3hrs	2.5 hrs	18 min	39 min
Limit of detection (LoD)	0.08 ng/ml	0.023 ng/ml	0.01 ng/ml	d 0.02 ng/ml
Limit of Quantification (LoQ)	0.16 ng/ml	0.06 ng/ml	0.03 ng/ml	d 0.08 ng/ml
Measurement range	0.16-22.5 ng/ml	0.06-11.6 ng/ml	0.01-23.0 ng/ml	0.02-24.0 ng/ml

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Supplemental Table 2: Patient characteristics and pregnancy outcomes of study group

	median	**95% CI**	**min-max**
Number of patients/cycles	83		
Age of women (years)	33	32-34	24-44
Antral follicle count (AFC)	12	9-13	1-35
Number of acquired cumuluses	11	8-13	1-35
Number of MII	6	6-7	0-26
Number of embryos transferred	1	1-1	0-2
Clinical pregnancy rate per cycle started (n, %)	40/83 (48.2%)		
Biochemical pregnancy rate per cycle started (n, %)	48/83 (57.8%)		
Ectopic pregnancy rate per cycle started (n, %)	0/83 (0%)		
Miscarriage rate per cycle started (n, %)	2/83 (2.4%)		

Supplemental Table 3: Summary characteristics of AMH measurements for all four assays.

	ELISA assays		Automated assays	
	Ansh Labs AMH/MIS ELISA	Beckman Coulter AMH Gen II ELISA	Beckman Coulter AccessAMH assay	Roche Elecsys AMH
Median [ng/ml]	3.34	3.21	2.83	2.44
95% CI	2.054 – 4.390	2.253 – 4.344	1.896 – 3.609	1.782 – 3.010
Minimum	0.02	0.02	0.07	0.06
Maximum	22.06	22.00	13.82	13.62
25 - 75 P	1.330 – 5.842	1.372 – 5.642	1.193 – 4.755	1.140 – 4.202

Supplemental Table 4: Passing-Bablok regression equations comparing assay values

	ELISA assays		Automated assays	
	Ansh Labs AMH/MIS ELISA	Beckman Coulter AMH Gen II ELISA	Roche Elecsys AMH	Beckman Coulter Access AMH assay
Ansh Labs AMH/MIS ELISA	-	Slope: 1.041 (95%CI 0.994, 1.101) Intercept: -0.201 (95%CI -0.327, -0.076)	Slope: 1.454 (95%CI 1.379, 1.526) Intercept: -0.368 (95%CI -0.507, -0.256)	Slope: 1.348 (95%CI 1.262, 1.437)x Intercept: -0.407 (95%CI -0.572, -0.234)
Beckman Coulter AMH Gen II ELISA	Slope: 0.960 (0.908, 1.006) Intercept: 0.193 (0.076, 0.297)	-	Slope: 1.372 (95%CI 1.327, 1.416) Intercept: -0.119 (95%CI -0.018, -0.050)	Slope: 1.280 (95%CI 1.242, 1.320) Intercept: -0.164 (95%CI -0.262, -0.086)
Roche Elecsys AMH	Slope: 0.688 (0.656, 0.725) Intercept: 0.253 (0.186, 0.333)	Slope: 0.729 (0.706, 0.754) Intercept: 0.087 (0.037, 0.128)	-	Slope: 0.943 (95%CI 0.893, 0.972) Intercept: -0.037 (95%CI -0.094, 0.026)
Beckman Coulter Access AMH assay	Slope: 0.742 (95%CI 0.696, 0.792) Intercept: 0.302 (95%CI 0.185, 0.398)	Slope: 0.781 (95%CI 0.758, 0.805) Intercept: 0.128 (95%CI 0.070, 0.198)	Slope: 1.061 (95%CI 1.028, 1.120) Intercept: 0.039 (95%CI -0.030, 0.096)	-

Passing-Bablok Regression equations where y=slope(x) + intercept, where y is represented in vertical column and x horizontal columns

Slope and Intercept are provided with 95% Confidence Interval (95%CI).

Supplemental Table 5: Area under the receiver operating characteristic curves (95%CI) for prediction of (A) biochemical pregnancy and (B) clinical pregnancy.

	ELISA assays		Automated assays	
	Ansh Labs AMH/MIS ELISA	Beckman Coulter AMH Gen II ELISA	Roche Elecsys AMH	Beckman Coulter Access AMH assay
Biochemical Pregnancy	0.64 (0.52, 0.77)	0.63 (0.51, 0.77)	0.63 (0.51, 0.76)	0.65 (95CI 0.53, 0.78)
Clinical pregnancy	0.63 (0.51, 0.75)	0.61 (0.49, 0.73)	0.62 (0.50, 0.74)	0.63 (0.51, 0.76)