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Comparison of the second-generation Beckman Coulter IVD and first-generation AnshLabs ELISA assays for anti-Müllerian hormone in patients undergoing IVF treatment

Porównanie skuteczności testu drugiej genereacji Beckman Coulter IVD oraz testu pierwszej generacji AnshLabs ELISA w ocenie surowiczych stężeń hormonu AMH u pacjentek poddawanych leczeniu za pomocą zapłodnienia pozaustrojowego in vitro

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Abstract

Objectives: *Ovarian reserve is the main factor influencing the efficacy of infertility treatment. Currently, the anti-Müllerian hormone is the main indicator of the ovarian reserve and has a wide spectrum of clinical importance. It achieved a high clinical value right after the introduction of the first commercial AMH assays in 2005. Lack further research and development of the tests and monopoly on their production have led to a significant reduction of their quality, resulting in lowered veracity and usefulness. Therefore, we searched for an alternative to the Beckman Coulter assay. The objective of the study was to draw a comparison between the commonly used second-generation assay by Beckman Coulter and the ultra-sensitive first-generation assay by AnshLabs.*

Materials and methods: *Serum samples (n=520) were collected from female patients undergoing routine AMH evaluation before entering an IVF program. We chose samples of patients with the lowest correlation between the AMH serum level and response to stimulation. The AMH serum levels of the patients were examined using two AMH tests, the second-generation assay by Beckman Coulter and the first-generation assay by AnshLabs. Precision and accuracy of both methods were determined and the results of AMH serum levels of 130 patients were correlated with the number of: antral follicles (AFC), follicles after stimulation, and the obtained cumulus cells.*

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Results: *Both, precision and accuracy of the compared methods were highly satisfactory. The coefficients of variation obtained in the study conducted on two different levels of control material were lower than 12% and the load did not exceed 9%. The study proved that both of the methods yielded comparable results. The coefficient of variation between the first-generation and the second-generation AMH assays was 0.871.*

Conclusion: *Both methods might be applied in the evaluation of the ovarian reserve. The first- and second-generation assays show comparable correlation with the clinical effects of stimulation, however, it seems that first-generation assays are a better alternative to the unstable second-generation kits. The results from the first-generation assays are distributed on a wider range, which facilitates clinical interpretation.*

Key words: **anti-Müllerian hormone** / **first-generation assay** / **second-generation assay** /

Streszczenie

Cel pracy: *Ocena rezerwy jajnikowej jest głównym czynnikiem wpływającym na skuteczność leczenia niepłodności. Obecnie jednym z głównych wykładników rezerwy jajnikowej jest hormon antymüllerowski (AMH), który zyskał swoje szczególne znaczenie w 2005 roku tuż po prezentacji pierwszego komercyjnego zestawu laboratoryjnego do jego oznaczania w surowicy. Brak rozwoju badań nad produktem i monopolizacja jego produkcji doprowadziły do znacznego obniżenia jakości testu, a tym samym znacznie obniżając wiarygodność wyników i ich przydatność. Dlatego też, poszukujemy alternatywy dla testu Beckmana Coultera.*

Celem pracy było porównanie skuteczności testu pierwszej generacji AnshLabs z testem drugiej genereacji Beckman Coulter w ocenie surowiczych stężeń hormonu antymüllerowskiego u pacjentów w trakcie leczenia za pomocą zapłodnienia pozaustrojowego in vitro.

Materiał i metodyka: *Próbki surowicy krwi do oznaczeń AMH (n=520) zostały pobrane od pacjentek przed rozpoczęciem kontrolowanej stymulacji jajników w programie zapłodnienia poza ustrojowego in vitro. Celem wiarygodniejszej analizy, do porównania wybrane zostały jedynie próbki surowicy krwi od pacjentek z niską zależnością między poziomem AMH a odpowiedzią na stymulację. Ocena surowiczych stężeń AMH dla testu Beckman Coulter oraz testu AnshLabs wykonana została metodą dwukrotnych powtórzeń.*

Precyzja i dokładność obu metod została wyznaczona, a wyniki badań wykazały dodatnią korelację pomiędzy surowiczymi stężeniami AMH w surowicy krwi 130 pacjentek a liczbą pęcherzyków antralnych w jajnikach, ilością pęcherzyków po stymulacji oraz liczbą uzyskanych kumulusów.

Wyniki: *Zarówno precyzja jak i dokładność obu zastosowanych metod była zadowalająca. Współczynniki zmienności uzyskane w badaniu przeprowadzone na dwóch różnych poziomach grupy kontrolnej były niższe niż 12%, a obciążenie nie przekraczało 9%. Badania wykazały, że obie metody dały podobne wyniki, a współczynnik zmienności między testem pierwszej generacji i testem drugiej generacji był na poziomie 0.871.*

Wnioski: *Obie metody mogą być stosowane w ocenie rezerwy jajnikowej. Zestawy obu generacji wykazują porównywalną korelację z klinicznymi efektami stymulacji, jednak zestawy pierwszej generacji wydają się być lepszą alternatywą wobec niestabilnych zestawów drugiej generacji. Dzięki szerszemu zakresowi wyników w przypadku użycia testów pierwszej generacji łatwiejsza jest ich interpretacja kliniczna.*

Słowa kluczowe: **hormon anti-müllerowski** / **testy pierwszej generacji** /
/ **testy drugiej generacji** /

Introduction

Oocyte quality depends on maternal age and ovarian reserve but it shows no direct correlation. Precise assessment of a woman's ovarian reserve has constituted the main problem in medical treatment of infertility for many years. At present, a variety of diagnostic tests are used in Poland to evaluate the ovarian reserve. The most common are: serum levels of follicle-stimulating hormone (FSH), estradiol, inhibin B and anti-Müllerian hormone (AMH) in the early follicular phase, as well as antral follicle count (AFC) [1]. Nowadays, AMH level is considered to be the best marker of the ovarian reserve. The anti-Müllerian hormone, also known as the Müller inhibiting substance (MIS), is a dimeric glycoprotein belonging to the transforming growth factor-β (TGF-β) family [1-3].

The AMH serum level reflects the pool of small ovarian follicles – when the amount of growing follicles decreases, the AMH serum level also falls. [4]. Its expression is the highest in the antral and pre-antral follicles. Through its paracrine activity, AMH inhibits both, growth stimulated by FSH and development of other primary follicles, while ensuring the selection of the dominant follicle [5-8].

Constant level of AMH during the menstrual cycle makes it a unique endocrinal parameter in measuring female gonad function, allowing to estimate patient fertility rate and to approximate the time of conception [4, 9, 10]. Even undetectable levels of AMH do not exclude the possibility of conceiving [11, 12].

Currently, evaluation of AMH is important in assisted-reproduction methods, where the hormone level is used to predict

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patient response to stimulation [13-16]. However, AMH might be used for many other reasons, for example as a diagnostic criterion for the differentiation of secondary amenorrhea. AMH concentration significantly increases in women with polycystic ovary syndrome (PCOS) and is significantly reduced in premature expiration of the ovarian function (POF), but remains unchanged in hypogonadism and hyperprolactinemia [9,17]. AMH might also be used to assess the level of damage caused by ovarian surgery or chemotherapy [18,19]. Furthermore, serum AMH concentration might also predict an estimated time to menopause in a patient [20-22].

AMH serum level may be determined using a variety of assays: the original research assays, the first-generation Diagnostic Systems Laboratories (DSL) and Immunotech assays, or the second-generation Beckman Coulter assay, which combines the cross-species DSL antibodies according to Immunotech standards. Also, the new, fully automated AMH assay will be released soon by several companies. AMH molecules are found in the serum as partly digested dimers. Due to the molecular structure of AMH, the assays usually use immunochemical methods with different detection systems. The European market of diagnostics tools offers several AMH testing sets using the enzyme-linked immunosorbent assay (ELISA) technique, as well as the second-generation assay by Beckman Coulter, which is currently the most widely used.

Rapid commercialization of scientific methods has created standardization gaps in various products - in this case the AMH assays – which are difficult to bridge. Large companies take over smaller ones, what destroys competitiveness and often leads to reduced product quality. Evolution of the tests and monopoly on their production have led to reduced quality of sets, including reliability and usefulness of AMH assays. Variety of patterns, flexible selection of antibodies added to the reagents, and their susceptibility to interfering factors, are the reasons why sets differ in sensitivity, specificity, linearity, range, precision and accuracy. Ultimately, they make the comparison of the obtained results impossible. Reliable results of AMH levels are of utmost importance as incorrect results might lead to wrong decisions concerning further diagnosis and treatment strategies. The aim of our study was to compare the AMH levels measured with the use of two different assays (the second-generation Beckman Coulter set and the first-generation ultra-sensitive set by AnshLabs).

Material and methods

The analyzed material included 520 blood serum samples from patients undergoing routine AMH tests before starting an IVF Program (BC II IVD), between October 2012 and February 2013. We selected 130 serum samples of patients with the lowest and the highest ratio of the number of antral follicles to AMH for comparison. The average ratio was 9.2±2.28. The inclusion criterion was the location of the ratio in the 12.5% of the results in both extremes. Then, the determination of AMH serum levels from the same material was made with the first-generation test by AnshLabs. Correlations between AMH levels and the number of: antral follicles, follicles after stimulation, and the obtained cumulus cells were also examined.

Blood sampling was performed between day 1 and 5 of the cycle. The blood was collected aseptically into tubes with clotting activator, using a vacuum blood collection system (Vacutainer; Becton Dickinson). After clotting was complete, blood samples were centrifuged (10 min, 1500 x g) to obtain sera. The obtained sera were frozen at -80°C until analysis. AMH was measured by ELISA - firstly by sets of the second-generation of AMH Gen II ELISA Beckman Coulter (Beckman Coulter Inc., Brea, CA USA) and then by sets of the first-generation of ultrasensitive AMH/MIS ELISA by AnshLabs. Detection limits of the sets were 0.08 and 0.02 ng/ml, respectively (Table I).

In the first stage of the study, precision of the AMH tests was evaluated for two different control sera, supplied by the manufacturers (Controls 1 and 2 for Beckman Coulter, and CTR-I and -II for AnshLabs).

The AnshLabs control material was supplied lyophilized. Determination of AMH in the above-mentioned control material was carried out according to the Clinical and Laboratory Standards Institute protocol (CLSI). The tests were performed by determining the level of AMH twice in one day, at 2-hour intervals and in two samples. The total number of measurements for one of the analyzed levels of control material was 20. 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 [23].

Both sets of AMH Gen II ELISA Beckman Coulter and Gen I AnshLabs tests are "sandwich" type immunoassays. The calibrators, controls and samples are incubated in the wells that have been coated with anti-AMH antibody. After incubation and washing, an anti-AMH labelled by biotin is added to each well. After the second stage of the incubation and washing, horseradish peroxidase (HRP)-conjugated with streptavidin is added to the wells. After another incubation and washing, tetramethylbenzidine (TMB) is added to the wells. In the final stage of the test, a solution of the acid is added to stop the reaction. The level of binding of tracers to the base is determined by the absorbance measurement at dual wavelength at 450 nm and from 600 to 630 nm. The measured absorbance is directly proportional to the concentration of AMH in samples. The AMH calibrator set is used to draw a calibration curve of absorbance versus concentration of AMH, from which concentration can be calculated from the calibration curve.

In both cases, the AMH assay results are presented in units of 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 re-analyzed. Measuring ranges for reagent kits used, their functional sensitivity and analytical sensitivity are presented in Table I.

MedCalc 12.1.4.0 and Statistica ver. 10 (Tulsa OK, USA) were used to evaluate the results. Pearson correlation analysis and Passing-Bablok regression equation were used to estimate the relationship between the obtained results with different analyses. Student's t-test for dependent pairs was used to assess the significance of differences. Bland and Altman graphs were used to visualize the data scatter [24].

Results

Results of precision and accuracy of each AMH assay are shown in Table II. The obtained coefficients of variation for compared sets of analytical and evaluated AMH values were satisfactory. In each case, the analyzed CV value was less than

Table I. Characteristics of the compared methods.

	Measuring range (ng/ml)	Analytical sensitivity (ng/ml)
AMH Gen II ELISA Beckman Coulter	0.08 – 22.5	0.08
AMH/MIS ELISA AnshLabs	0.02 - 10.7	0.02

ELISA – enzyme-linked immunosorbent assay, AMH – Anti-Müllerian Hormone

Table II. The results of the precision and accuracy of both AMH assays.

Control sample	Nominal value	Precision					Correctness	
		average	min	max	SD	CV%	bias	bias %
AMH Gen II ELISA Beckman Coulter								
Control 1	2.9	2.86	2.51	3.11	0.22	7.7	-0.01	-1.4
Control 2	8.2	8.47	7.44	9.65	0.58	6.8	0.03	3.3
AMH/MIS ELISA AnshLabs								
CTR-I	1.2	1.09	0.84	1.3	0.12	11.1	-0.09	-9.0
CTR-II	3.7	3.5	2.89	4.0	0.41	11.7	-0.05	-5.3

CV – coefficient of variation, AMH – Anti-Müllerian Hormone, SD – standard deviation

Table III. The comparison of AMH values in both tests.

	<35 years	35-37 years	38-39 years	>39 years
AMH Beckman	1.95 ± 2.00 (69)	1.42 ± 1.50 (23)	1.52 ± 2.13 (17)	1.08 ± 0.98 (21)
AMH AnshLabs	3.25 ± 3.02 (69)	2.93 ± 3.05 (23)	1.89 ± 1.57 (17)	2.27 ± 2.59 (21)
p-value[a]	< 0.0001	0.0004	0.2807	0.0059

Data are presented as means ± standard deviation (SD) (n- number of tested samples)
a Student's t-test.

Table IV. The correlation between AMH levels and the number of antral follicles (AFC), number of follicles after stimulation and number of acquired cumuluses.

Age groups		AFC	amount of follicles after stimulation	number of acquired cumuluses
<35 years	AMH Beckman	0.59	-0.26	-0.33
	AMH AnshLabs	0.64	-0.25	-0.34
35-37 years	AMH Beckman	0.74	-0.3	-0.42
	AMH AnshLabs	0.79	-0.33	-0.44
38-39 years	AMH Beckman	0.64	-0.03	-0.39
	AMH AnshLabs	0.52	0.22	-0.05
>39 years	AMH Beckman	0.68	0.23	0.04
	AMH AnshLabs	0.76	0.39	0.18

12%. For both methods compared, correctness was satisfactory - in all of the analyzed cases the load did not exceed the value of 9%.

Correctness of AMH results obtained by AMH Gen II ELISA Beckman Coulter assay was higher than by AMH/MIS ELISA AnshLabs assay. Comparative results of both AMH tests, performed on the same material and at the same time, are summarized in Table III.

Average values for specified levels of AMH were not comparable with each other: they were lower for Beckman Coulter assays than for AnshLabs assays. Student's t-test showed no statistically significant differences between the averages only in the group of women aged 38-39 years. Pearson correlation coefficient calculated for the relationship between AMH levels obtained by the compared analytical methods was high (0.871), and the Passing-Bablok regression equation was as follows:

$AMH_{(AMH/MIS\ ELISA\ AnshLabs)} = 0.6168 + 1.3512 \cdot AMH_{(Gen\ II\ ELISA\ Beckman\ Coulter)}$ (Figure 1). In both cases, a linear relationship was presented. Dispersion of the results obtained with both methods is presented in the Bland-Altman graphs (Figure 2).

Correlation between AMH levels and the number of antral follicles, follicles obtained after stimulation and acquired cumulus cells was also examined (combination of the results – Table IV). The first-generation assay showed a slightly higher coefficient of correlation with clinical status of the patients than the second-generation assay.

Discussion

Ensuring good quality of AMH evaluation is the basis of good research in the field of women's ovarian reserve. This determination should be sufficiently sensitive, analytically and functionally, and the spectrum of the measured concentrations should be relevant to those that are most common in the patient population. Our findings demonstrated that the precision of both, AMH/MIS ELISA by AnshLabs and AMH Gen II ELISA by Beckman Coulter met these requirements. Although the nominal values of AMH in control material supplied by the manufacturers were slightly different from the assumed values (which is particularly evident in the case of AnshLabs control material), the obtained coefficients of variation in each of the analyzed cases were lower than 12%. This allows us to conclude that both methods offer adequate precision of determination and the information about the efficacy of the determination showed in methodical brochures is consistent with the results obtained in our study.

The narrower range of AMH linearity for the AMH/MIS ELISA by AnshLabs in comparison with the AMH Gen II ELISA by Beckman Coulter is of little, if any, practical significance. The percentage of samples above the measuring range was similar for both methods. The AMH/MIS ELISA AnshLabs assay is characterized by higher analytical sensitivity and functional sensitivity (Table I – manufacturer's data), so it is able to detect lower concentrations of AMH, which can be particularly useful in the assessment of the ovarian reserve.

Although correctness of AMH determination by AMH Gen II ELISA set from Beckman Coulter was slightly better than AMH/MIS ELISA set from AnshLabs, it seems unlikely that the obtained differences might affect the interpretation of the acquired results in a statistically significant way. Regardless of the analytical method, the result load was lower than 9%. The obtained data clearly showed that correctness of the results was satisfactory and the load factor was much lower than expected by the experts on laboratory quality management, where the upper range of permissible error ranges from 15 to 20%.

Comparison of AMH tests made in the sera of patients undergoing routine determination of AMH before IVF programs also confirms usefulness of both methods in terms of analysis. Both methods were characterized by a linear relationship with a high correlation coefficient (0.871). The results acquired by Beckman Coulter assay were much lower than those obtained by the AnshLabs assay, but the calculated conversion coefficient from one test to another is not constant. Both assays could be used for sera which are not properly preserved. The laboratory procedures are comparable and not different from other ELISA methods. Also, their cost is comparable.

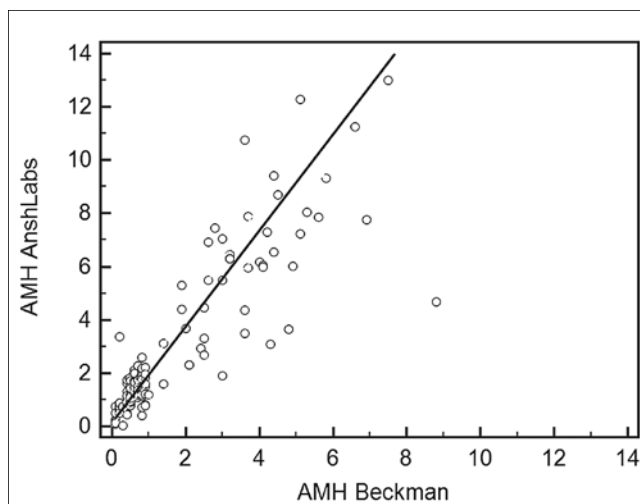

Figure 1. Passing and Bablok regression analysis between AMH concentrations (ng/ml) obtained with AMH/MIS ELISA AnshLabs and Gen II ELISA Beckman Coulter assays for 130 serum samples. Linear regression analysis results were r = 0.871; P < 0.0001; AMH (AnshLabs) = 0.6168 + 1.3512 · AMH (Beckman Coulter).

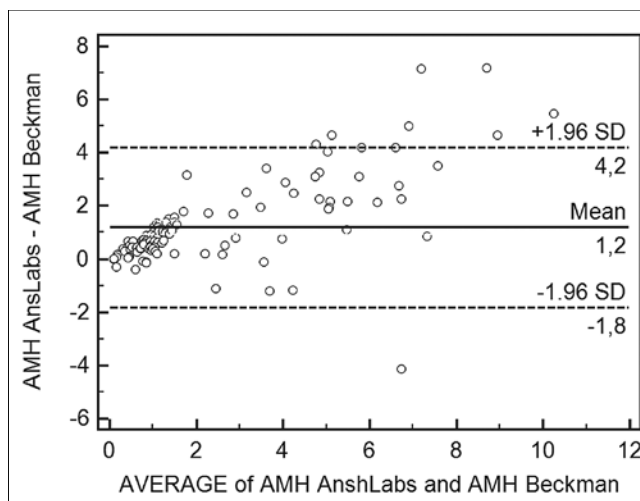

Figure 2. Bland-Altman plot of the agreement between AMH/MIS ELISA AnshLabs and Gen II ELISA Beckman Coulter assays (solid line = mean difference; dotted lines = 95% CI).

AMH not only has a strong relationship with the number of antral follicles, but it is also the best marker amongst other typical biomarkers correlating with AFC [25]. There is an ongoing debate about the usefulness of AMH and AFC in ovarian response to stimulation for IVF. The correlations between AMH and response to stimulation vary for different kits. AFC depends on the observers, their experience and ultrasound machines used. That is why the AMH, after finding a stable measurement system, will be more comparable for clinicians and scientists.

Our findings of poor correlation between both assays and the number of the received follicles and cumulus cells after stimulation should not be interpreted as the assessment of quality of AMH assays in general, as we selected the extremes of the results for our analysis. We wanted to compare both assays and we found similar results. These results might lower the applicability

of AMH kits in predicting the success of controlled ovarian stimulation in IVF treatment . Different correlation of each AMH assay and effects of stimulation – negative for younger and positive for older subjects - are caused by differences in patient population. As we chose the extremes of the results from both sides, we got high AFC and AMH for younger and low for both, younger and older subjects. Low AFC facilitates a more precise count of the follicles, whereas in case of high AFC the precision is lower, what can influence the results.

AMH level is also significantly correlated with the number of follicles obtained after stimulation and the number of oocytes acquired in the IVF cycle. Several studies showed a possibility of evaluating the ovarian reserve based on the AMH serum level, and predicting the risk of low, average or excessive ovarian response to stimulation [14,15,26]. Also, some authors report a relationship between AMH serum level and approximate the rate of live births [10,27]. Identification of higher response to stimulation in a patient allows for more effective treatment and prevention of the ovarian hyperstimulation syndrome (OHSS) [28-29].

Conclusions

Our study aimed to evaluate correlation between serum levels of AMH determined by the first and the second-generation assays, and clinical parameters for ovarian response, i.e. the number of antral follicles, follicles obtained after stimulation, and acquired cumulus cells. Both methods might be applied in the evaluation of the ovarian reserve in IVF treatment. The first-generation assay showed comparable correlation between the measured serum AMH level and the clinical features, therefore it seems to be a good alternative to the unstable second-generation assays. The results from the first generation assays are distributed on the wider range, which facilitates clinical interpretation.

Author Contributions

Conceived and designed the experiments: KL, WK, IM, BL. Analyzed the data: JL, EP, AB, MK. Wrote the paper and interpretation of results: EP, AL, KL, AK, PK. Reviewed all statistical analyses: JL. All authors critically reviewed and approved the final manuscript.

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