

New Sensitive Anti-Müllerian Hormone (AMH) ELISAs for Non-Human Primate, Rodent, Equine, Bovine, Canine and Other Species

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ABSTRACT

Objective: Development of specific and sensitive ELISA kits to quantify AMH in sera of different species.

Relevance: Anti-Müllerian hormone (AMH) is a 140-kDa dimeric glycoprotein hormone belonging to the transforming growth factor- β (TGF- β) superfamily. Cleavage at the monobasic site generates 110-kDa N-terminal and 25-kDa C-terminal homodimers, which remain associated in a noncovalent complex. Recent studies have shown that the AMH C-terminal homodimer is much less active than the non-covalent complex, but almost full activity can be restored by associating the N-terminal pro-region, which re-forms a complex with the mature C-terminal dimer. The finding suggests that the AMH non-covalent complex is the active form of the protein.

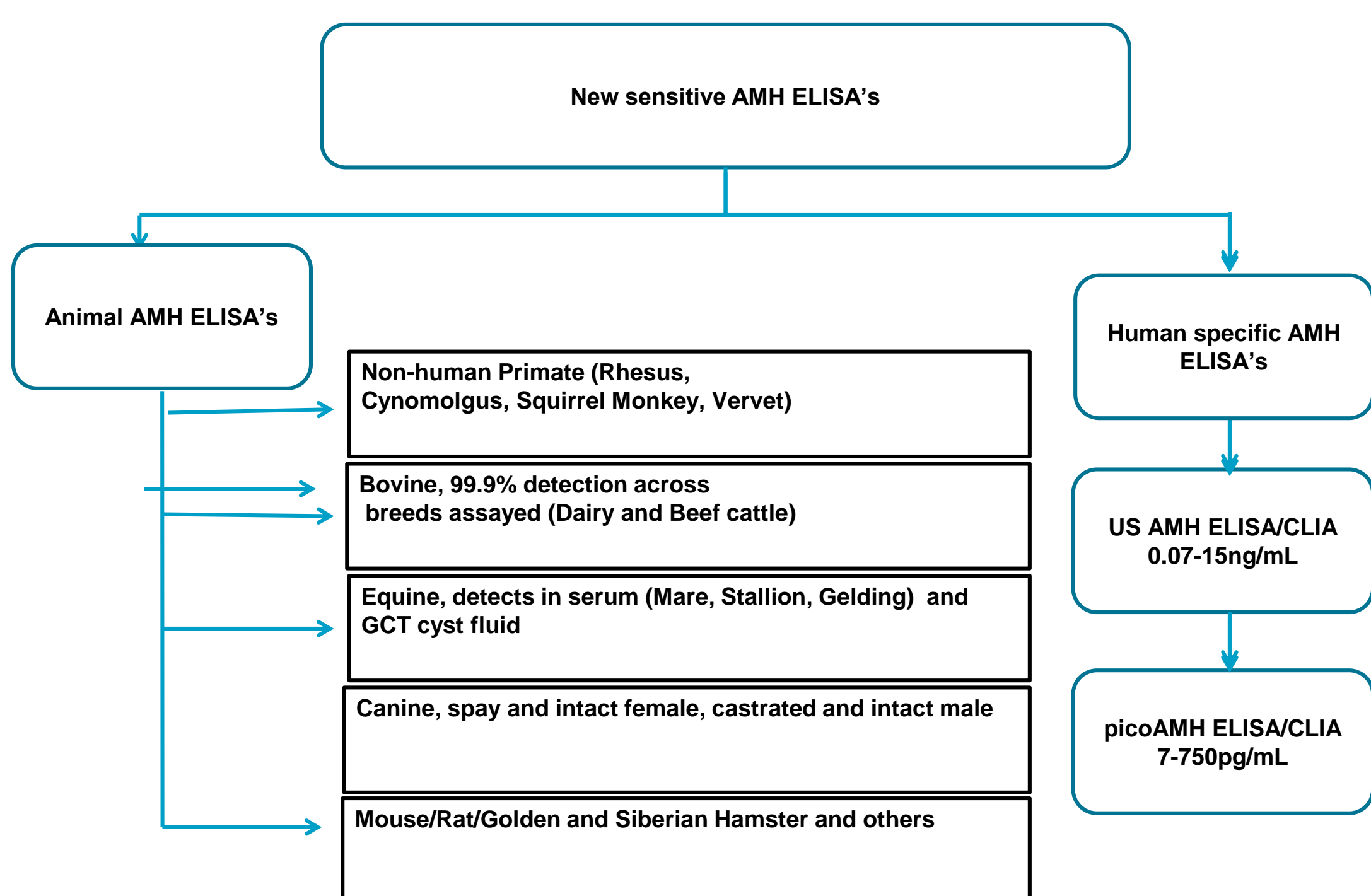
Methods: We have developed three-step, sandwich-type enzyme-immunometric microplate assays to measure species specific AMH levels in small samples sizes from 10-50 μ L of sera in less than 3.5 hours. Equine, bovine, rat, and non-human primate assays utilize species-specific AMH calibrators. The monoclonal antibody pairs used in the above AMH assays bind to the non-covalent AMH complex and do not detect other related members of TGF- β superfamily.

Validation: Ansh Labs Rat/Mouse and Equine/Canine AMH ELISAs showed excellent clinical concordance between ovariectomized versus cycling rats, spayed and intact female dogs, castrated and intact male dogs, geldings, stallions, mare sera and granulosa cell tumor (GCT) cyst fluid, respectively. The Rat/Mouse AMH ELISA also detects Golden and Siberian hamsters. Ultra-sensitive AMH ELISA detected AMH concentrations in the range of 0.1-12 ng/mL in Rhesus, Cynomolgus, Vervet, and Squirrel monkey sera. The enhanced specificity and analytical sensitivity (0.011 ng/mL) of the Bovine AMH ELISA resulted in greater than 90% detection rate in various dairy and beef cattle breeds. Imprecision calculated on three pooled sera over twenty-four replicates was 2.92% at 0.61 ng/mL, 2.54% at 1.26 ng/mL and 3.65% at 2.56 ng/mL. Dilution and spiking studies confirmed accuracy of AMH measurement and showed average recoveries between 90-110% for all assays.

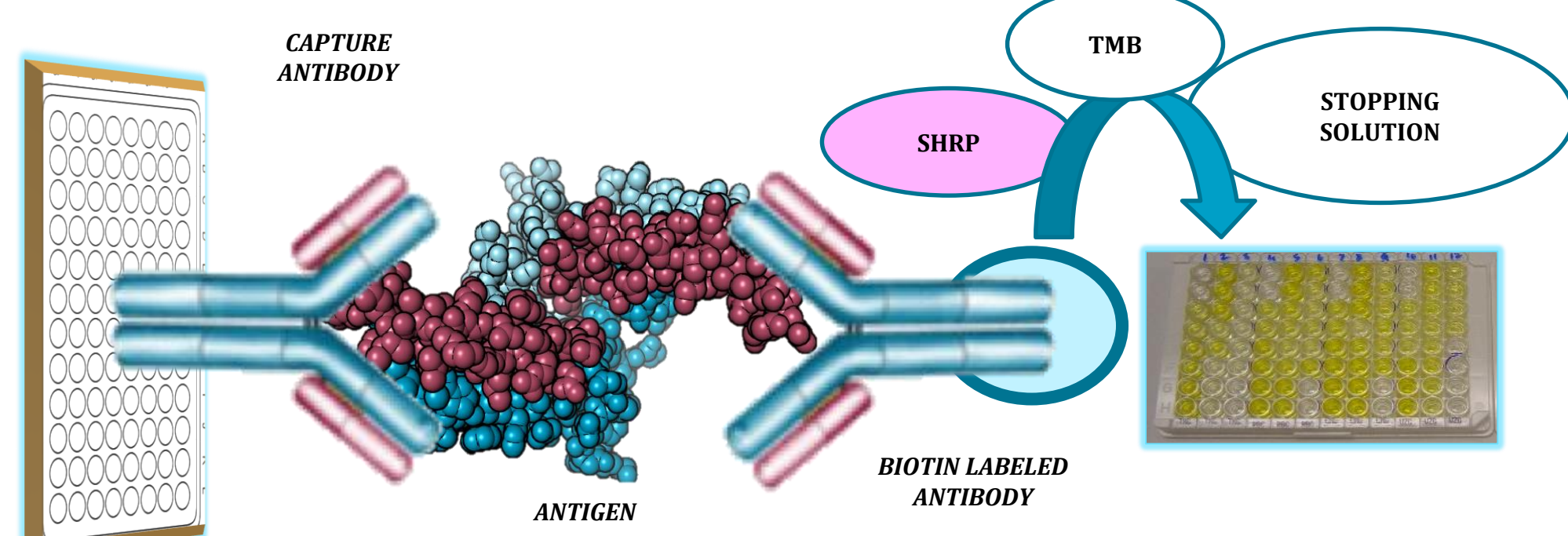
Conclusions: Specific, sensitive and reproducible AMH assays have been developed for the measurement of AMH in non-human primate, rodents, equine, bovine, canine and other species. The performance of these assays is ideal for investigation into the physiologic roles of AMH in different species.

INTRODUCTION

Recent finding suggests that the AMH non-covalent pro-mature complex is the active form of the protein. A three step sandwich assay to measure the associated form of AMH requires a high affinity antibody in the pro-region of species specific AMH. This has been a challenge due to the overall amino acid sequence homology between the pro-regions of mouse, rat, equine, bovine, canine, human and other species varies from 37-89% (Gene Bank NCBI). A series of comprehensive species immunoreactivity studies was performed over 73 pairs of AMH antibodies that resulted in very sensitive and clinically relevant AMH assays for different species and also generated numerous high affinity antibodies for immunostaining.



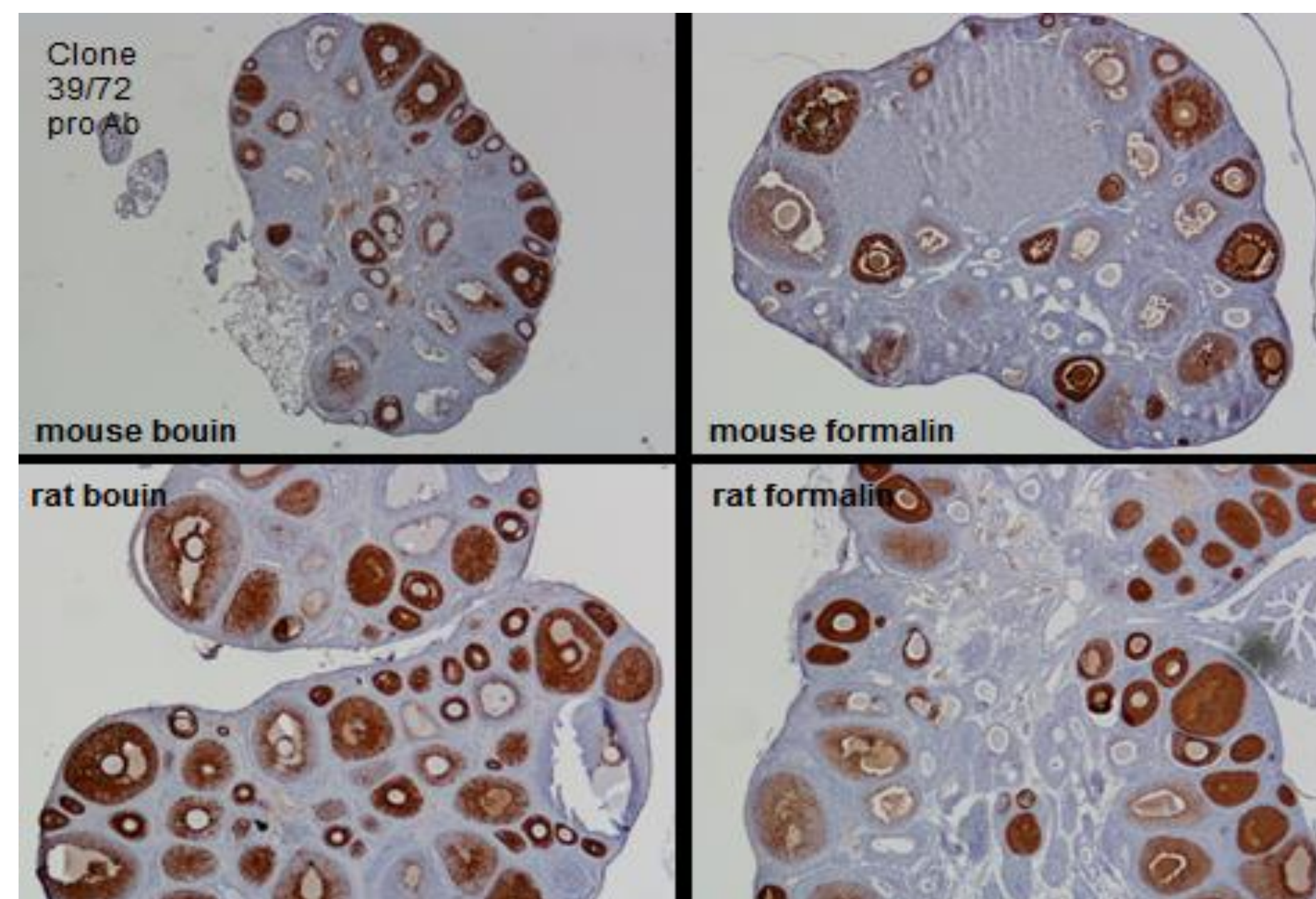
METHOD



RESULTS

Immunohistochemistry:

Antibody dilution 1:200 for rat and mouse Bouin's- and formalin-fixed.



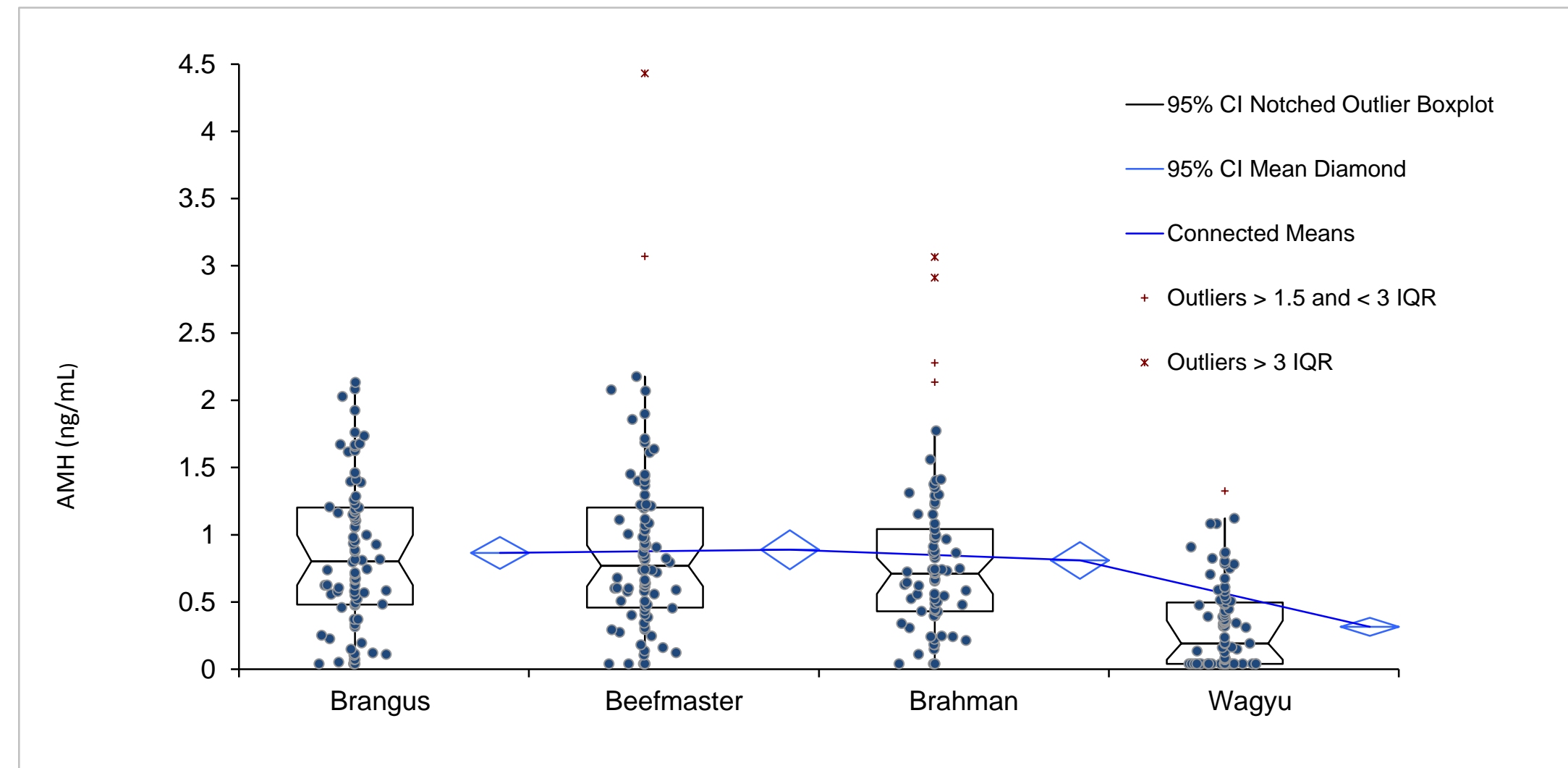
Results were measured as optical Density

Species Samples	23/32	23/37	23/24	24/32	24/37	8/10	17/15	17/37	36/37	36/33
Cal.A/Blank	0.18	0.06	0.11	0.05	0.05	0.10	0.09	0.04	0.05	0.05
hAMH, 0.073ng/mL	0.21	0.19	0.31	0.21	0.16	0.25	0.13	0.06	0.18	0.10
hAMH, 9ng/mL	1.53	4.34	4.51	4.62	4.43	4.57	2.92	1.23	4.37	3.30
Equine, 15ng/mL Cyst Fluid	3.03	4.68	0.10	0.11	0.11	0.09	3.12	4.57	4.68	4.12
Bovine Cal.-15-20ng/mL	1.76	4.44	0.09	0.05	0.05	0.09	3.25	1.10	4.26	4.04
Canine Intact, 1:10	1.46	0.05	0.10	0.05	0.05	0.09	3.24	0.04	0.03	2.27
Canine Spay	-	-	-	-	-	-	0.09	-	-	0.09
Rat AMH, ~15ng/mL	0.77	1.49	0.12	0.05	0.05	0.09	3.00	1.54	1.62	2.04
Mouse 1:10 dil	0.27	0.35	0.10	0.03	0.03	0.09	2.14	0.51	0.48	0.47
Squirrel Monkey F4, 1:30 dil	0.16	3.08	2.40	0.53	4.19	0.09	0.66	0.96	3.94	0.04
Vervet Monkey 2, 1:30 dil	0.18	0.05	0.58	0.45	0.04	0.09	0.12	0.03	0.02	0.09
Siberian Hamster	-	-	-	-	-	-	4.68	-	-	-
Golden Hamster	-	-	-	-	-	-	4.68	-	-	-

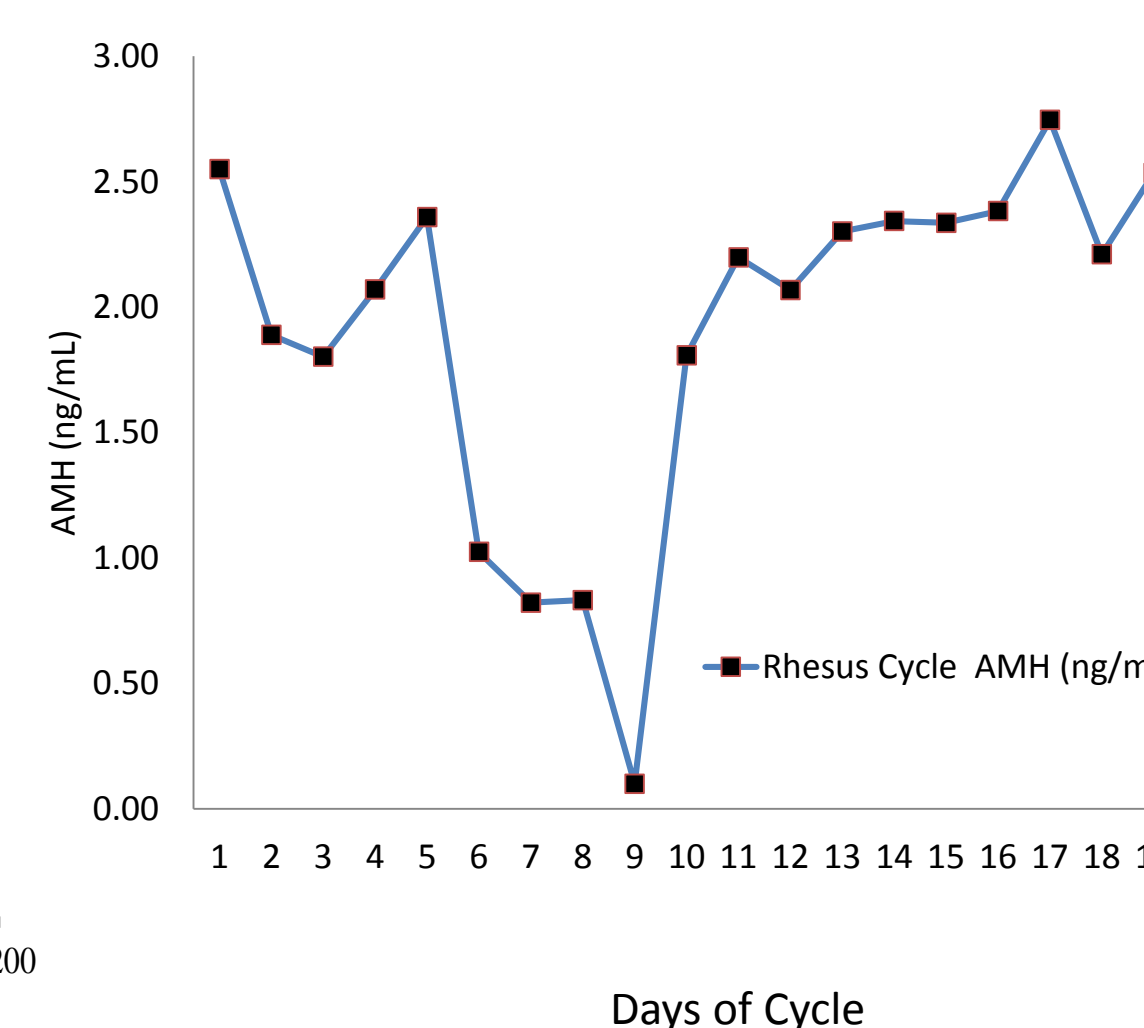
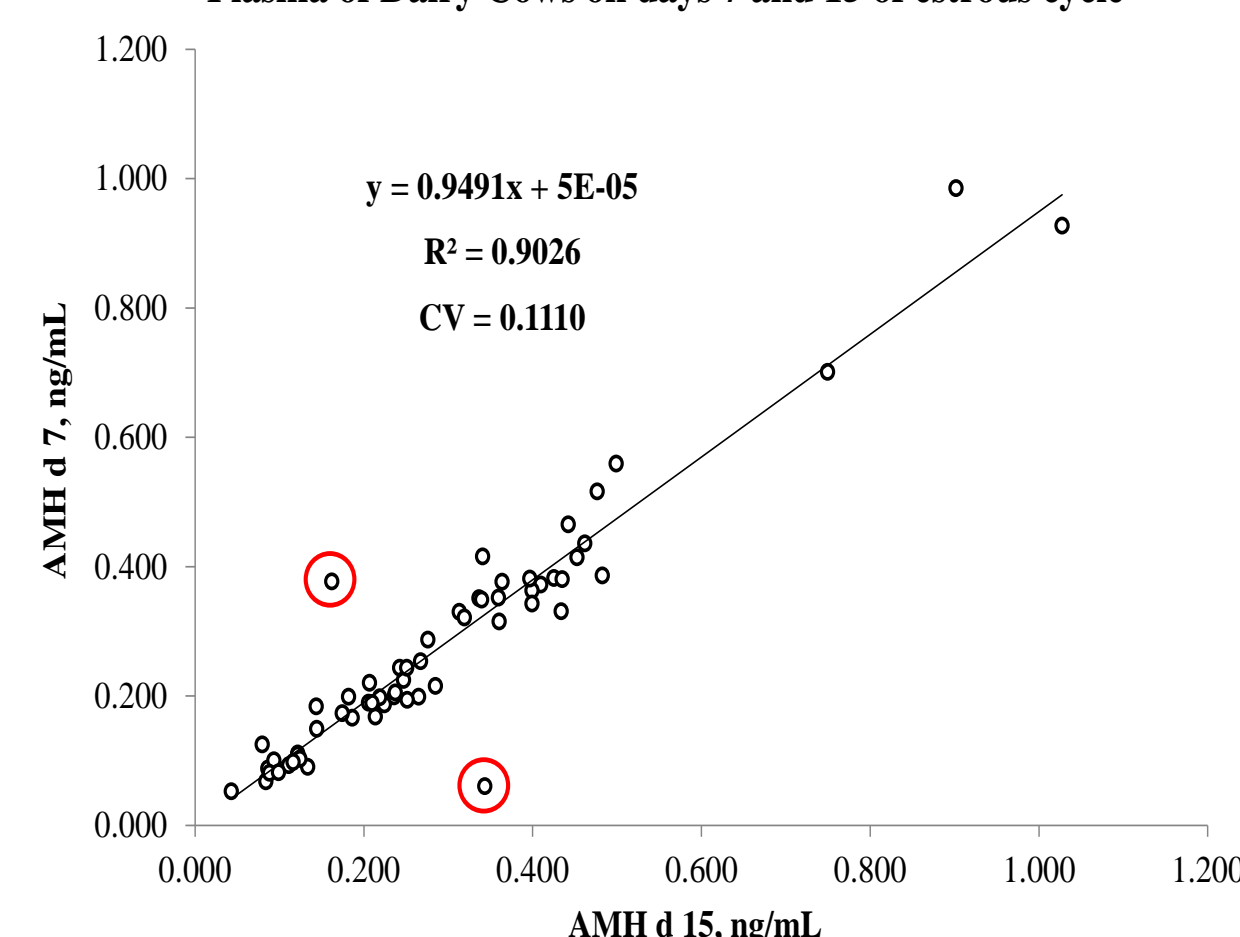
Bovine AMH Assay Calibration Curve

Well Number	Well Contents	Mean Absorbance	Conc (ng/mL)
Calibrators			
A1, A2	A	0.043	0
B1, B2	B/4	0.026	0.039
C1, C2	B/2	0.057	0.078
D1, D2	B	0.107	0.156
E1, E2	C	0.213	0.312
F1, F2	D	0.423	0.625
G1, G2	E	0.838	1.25
H1, H2	F	1.45	2.5
A3, A4	G	2.53	5.0

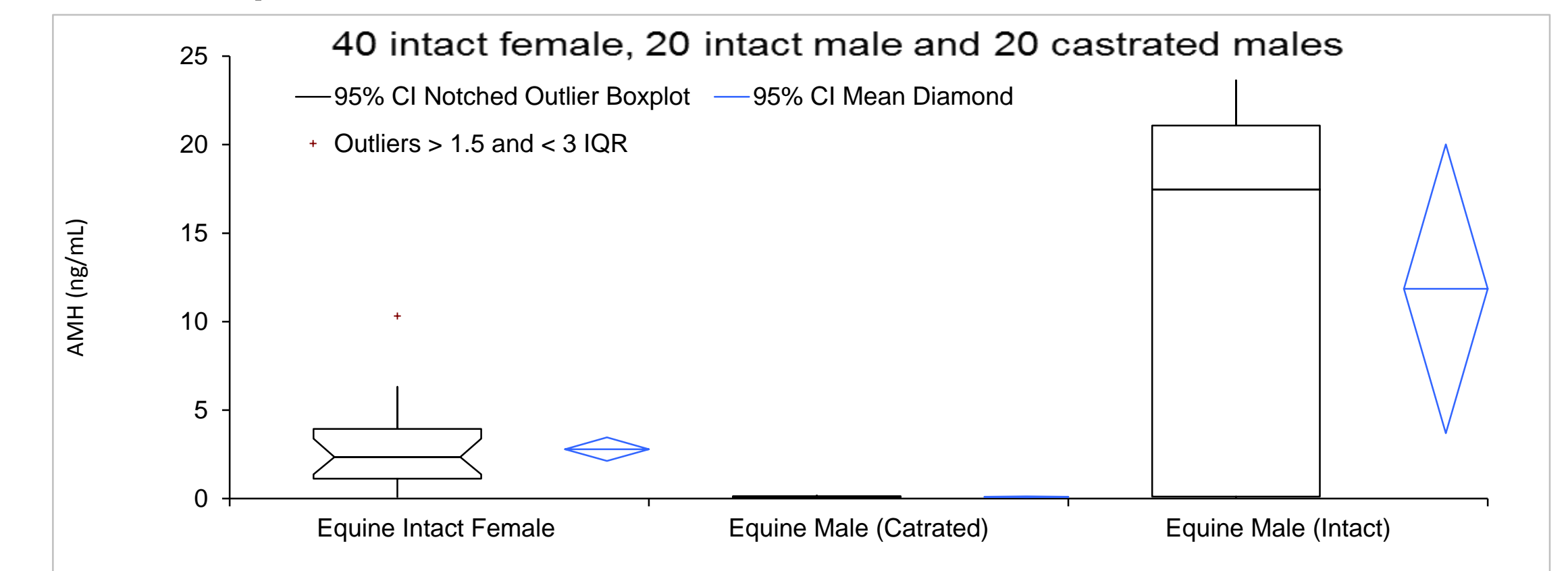
Bovine Breed and AMH



Regression Coefficient Between Concentrations of AMH in Plasma of Dairy Cows on days 7 and 15 of estrous cycle



AMH in Equine Intact Female, Male and Castrated Males:



Analytical Sensitivity

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 14 replicates of calibrator A (0 ng/mL) and calibrator B/4 (0.039 ng/mL) is 0.011 ng/mL

Imprecision:

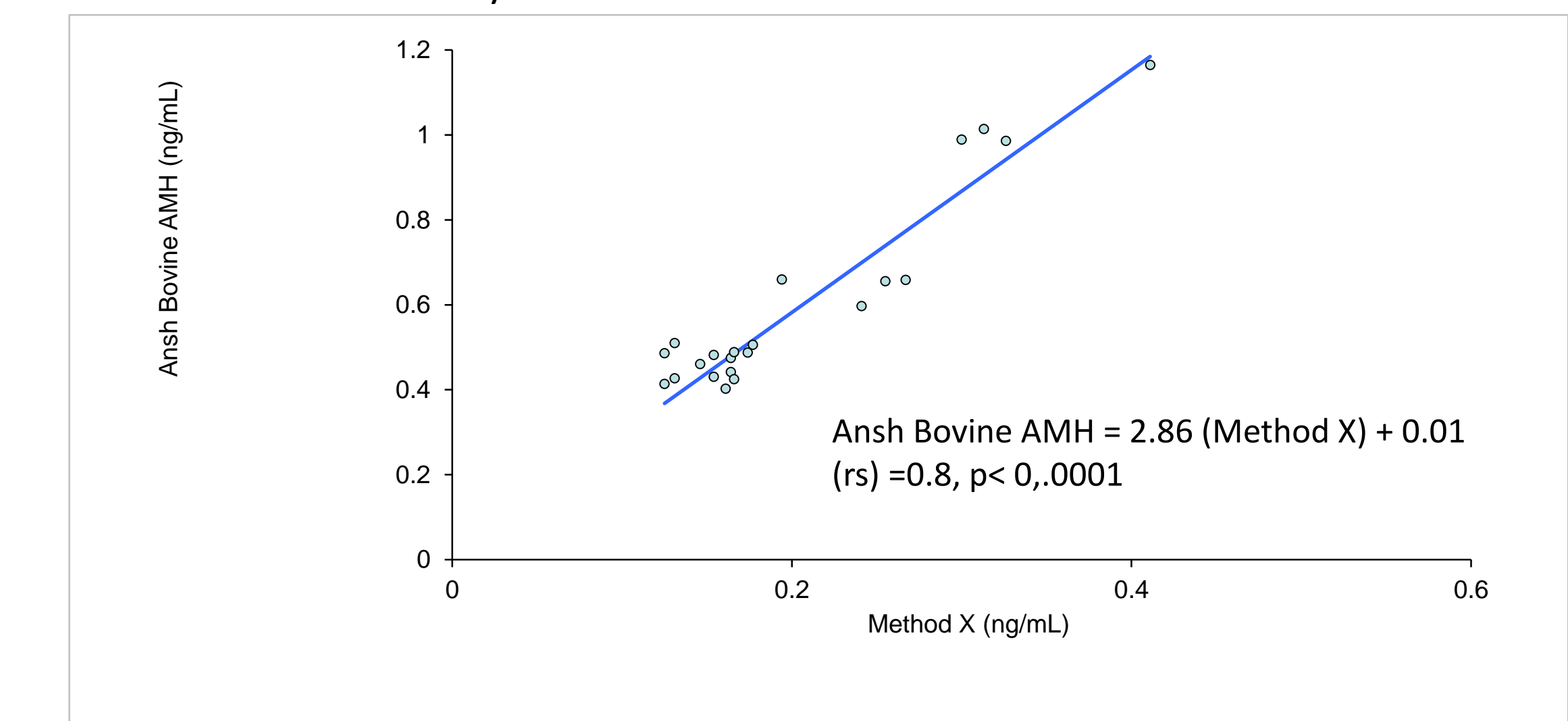
Sample	n	Mean Conc. (ng/mL)	SD	% CV
Pool-1	24	0.611	0.018	2.92
Pool-2	24	1.259	0.032	2.54
Pool-3	24	2.56	0.0933	3.65

Linearity of Dilution:

Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
Bovine	Neat	7.47	NA	NA
	1:2	3.73	3.73	100%
	1:4	1.87	1.97	106%
	1:8	0.93	0.98	105%
	1:16	0.47	0.51	109%
Squirrel Monkey	1:5	10.11	NA	NA
	1:10	5.05	5.18	103%
	1:20	2.53	2.49	99%
	1:40	1.26	1.41	112%
	1:80	0.63	0.59	94%
Equine	1:40	19.28	NA	NA
	1:80	9.64	9.85	102%
	1:160	4.82	4.75	99%
	1:320	2.41	2.47	102%
	1:640	1.21	1.27	105%
Canine	1:4	6.04	NA	NA
	1:8	3.02	3	99%
	1:16	1.51	1.47	97%
	1:32	0.75	0.75	100%
	1:64	0.37	0.39	104%
Hamster	1:10	2.2	NA	NA
	1:20	1.1	1.10	100%
	1:40	0.55	0.68	124%
Rat	Neat	9.93	NA	NA
	1:2	4.97	4.78	96%
	1:4	2.48	2.21	89%
	1:8	1.24	1.04	84%

Method Comparison

N=139 animals tested. 117 case were excluded as samples were not detected in another commercial assay.



CONCLUSIONS

Highly sensitive, reliable and easy-to-run microplate AMH assays have been developed to measure AMH in serum and other biological fluids of different species.

The assays show excellent analytical performance and measure AMH levels in healthy populations with <5% CV.

ACKNOWLEDGEMENTS

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