CHAPTER IV
RESULTS

Demographic information of samples

The demographic data of samples were calculated in topic of breed, occupation, and age of all subjects used. Breeds of subjects in this research consisted of Thoroughbred, Lipizanner, Dutch warmblood, and mixed breed. The major horse breed was Thoroughbred (95.97%) as shown in table 6. Various occupation of horses were shown in table 7. Mostly occupation of these horses was racing (61.74%) following by retired horse (16.12%) and foals that have no occupation (15.43%). The last one was riding horse (6.71%). The means and standard deviations of horse age in study A was shown in table 8. In OA group of the study B, all horses were racing Thoroughbred. There was no significant difference in age ($p < 0.05$) between non-OA group and the OA group (table 9). The number of non-OA group and OA in study B was shown in table 10.

Table 6 Breed of horse samples

Breed	n	Percent
Thoroughbred	143	95.97
Dutch warmblood	2	1.34
Lipizanner	1	0.67
Mixed breed	3	2.01
Total	149	100

Table 7 Occupation of horse samples

Occupation	n	percent
Foals (no occupation)	23	15.43
Racing horse	92	61.74
Riding horse	10	6.71
Retired horse	24	16.12
Total	149	100

Table 8 Mean and standard deviation of age of each normal horse group.

Age	n	Percent	Mean of age ± SD
Newborn - < 2 y	25	16.78	0.33 ± 0.46
2 y – < 5 y	25	16.78	2.84 ± 0.85
5 y – < 9 y	29	19.46	6.24 ± 1.21
9 y – < 15 y	33	22.15	11.84 ± 1.92
≥ 15 y	37	24.83	16.40 ± 1.72
Total	149	100	

Table 9 Mean and standard deviation of age of non-OA and OA horse

Assay	Age of non-OA (n)	Age of OA (n)	Significance
3B3	4.67 ± 2.00 (54)	5.05 ± 1.94 (22)	NS
WF6	4.67 ± 2.00 (54)	5.05 ± 1.89 (23)	NS
HA	4.67 ± 2.02 (52)	5.05 ± 1.96 (21)	NS

NS = non significance ($p>0.05$)

Table 10 Number of horse in non-OA and OA group

Group	n	Percent
Non-OA	54	70.13
OA	23	29.87
Total	149	100

Table 11 Laboratory quality control

Parameter	3B3	WF6	HA
Intra-assay %CV	7.17	6.64	12.55
Inter-assay %CV	17.52	9.2	25.05

Optimization of competitive inhibition ELISA

Optimization of 3B3 assay

From previous studies, it was suggested that 3B3(+) epitope was expressed when predigested cartilage or synovial fluid with chondroitinase ABC (Caterson, Griffin et al. 1990). In this thesis, 0.1 U/ml chondroitinase ABC was used to digest horse serum for overnight at 37°C before assayed. Other reagents used in this assay were shown in

table 5. The absorbance standard curve for 3B3 assay was shown in Figure 11. The range of detectable concentration was 4.53-2000 ng/ml. The intra- and inter-assay precisions were shown in table 11.

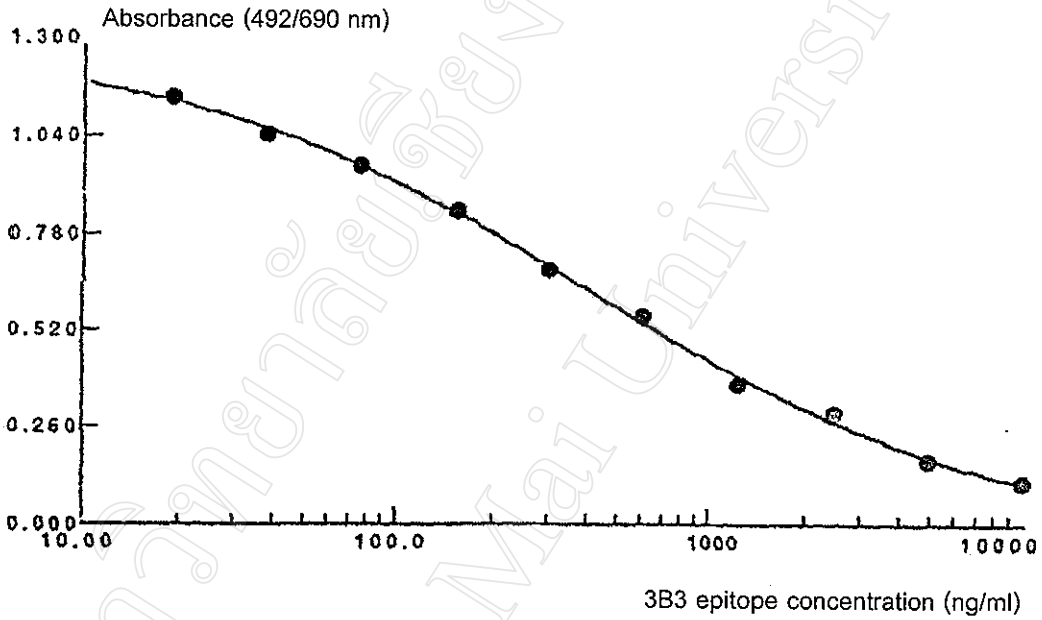

Figure 12 Typical 3B3 standard curve by competitive inhibition ELISA. Plate was coated with pig proteoglycan (chondroitinase core). Pig proteoglycan(chondroitinase core) was used as the standard competitor as described in table 5.

Optimization of WF6 assay

Monoclonal antibody WF6 recognized an epitope in native chondroitin sulfate chain. By competitive inhibition ELISA technique, WF6 mAb was applied to detect this epitope in horse serum using aggrecan (A1D1 fraction) as a standard competitor. The assay was developed by reagents that were shown in table5. Then the absorbance standard curve for WF6 was plotted in Figure 12. The range of detectable concentration was 19.53-10000 ng/ml. The intra- and inter-assay precisions were shown in table 11.

It was found that undiluted serum samples could not be used due to the greater absorbance than the control one. Therefore, serum was diluted two folds and then it gave the absorbance in standard curve.

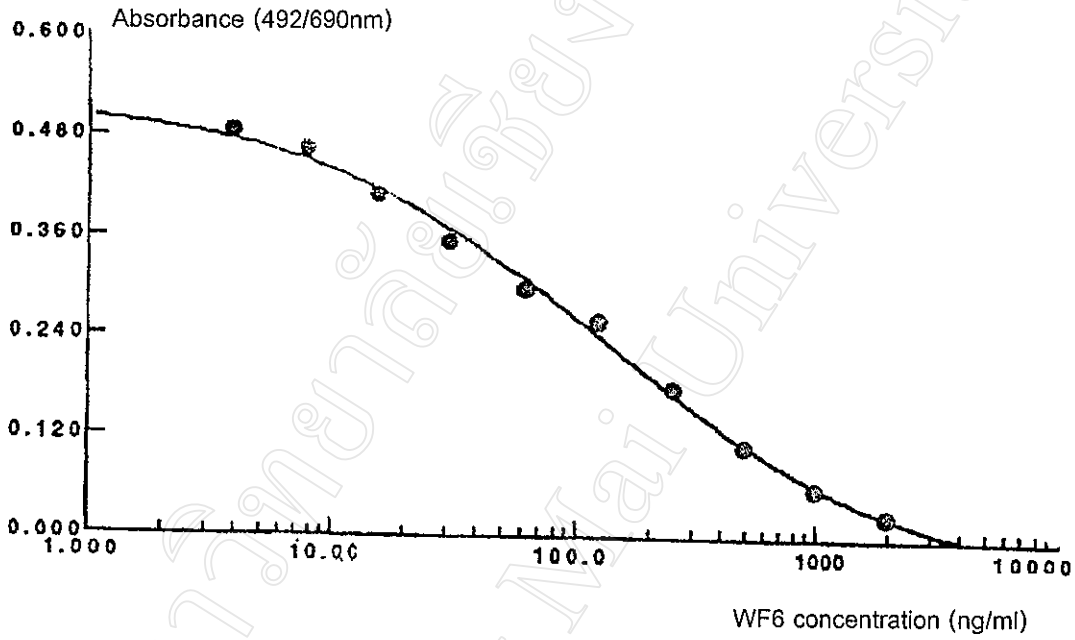

Figure 13 Typical WF6 standard curve by competitive inhibition ELISA. Plate was coated with shark proteoglycan (A1-fraction). Shark proteoglycan (A1D1 fraction) was used as the standard competitor as described in table 5.

Optimization of HA assay

HA assay also applied the principle of enzyme immunoassay but instead of monoclonal antibody, biotinylated-HABP (b-HABP) was used as the specific ligand for HA. From this method, the concentration of HA in serum could be detected. The reagents used in this assay were shown in table5. The absorbance standard curve for HA was shown in Figure 13. The range of detectable concentration was 19.53-10000 ng/ml. The intra- and inter- assay precision was shown in table 11.

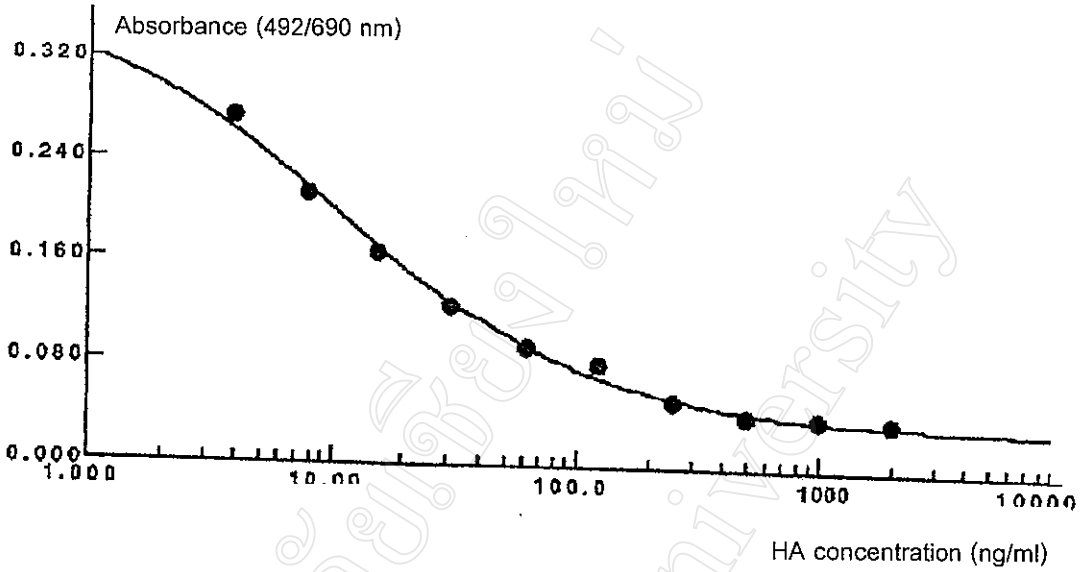

Figure 14 Typical HA standard curve for the competitive inhibition ELISA. Plate was coated with umbilical cord HA. Healon HA was used as the standard competitor as described in table 5.

Clinical performance

Study A: comparison ECM markers among age of normal healthy horses

In this study, concentration of HA and chondroitin sulfate epitopes (3B3 and WF6 epitope) were determined and compared in five categories depending on age of normal healthy horses. Two monoclonal antibodies, 3B3 and WF6 mAb, and b-HABP were used in detecting these ECM markers.

Determination of 3B3 epitope, WF6 epitope and HA were shown as mean and standard deviation in table 12 and as median in Figure 14, 15 and, 16. 3B3 epitope in newborn to less than two-year-old horses was significantly higher ($p<0.001$) when compare with other horse age groups. There was no different significance of 3B3 level among horse aged more than two years old (Figure 14). There was no different significance in WF6 concentration at any age groups (Figure 15). Analysis of HA showed that horse at newborn to less than five years old have a significantly higher ($p<0.001$) HA level than in more than or equal five-year-old horses (Figure 16).

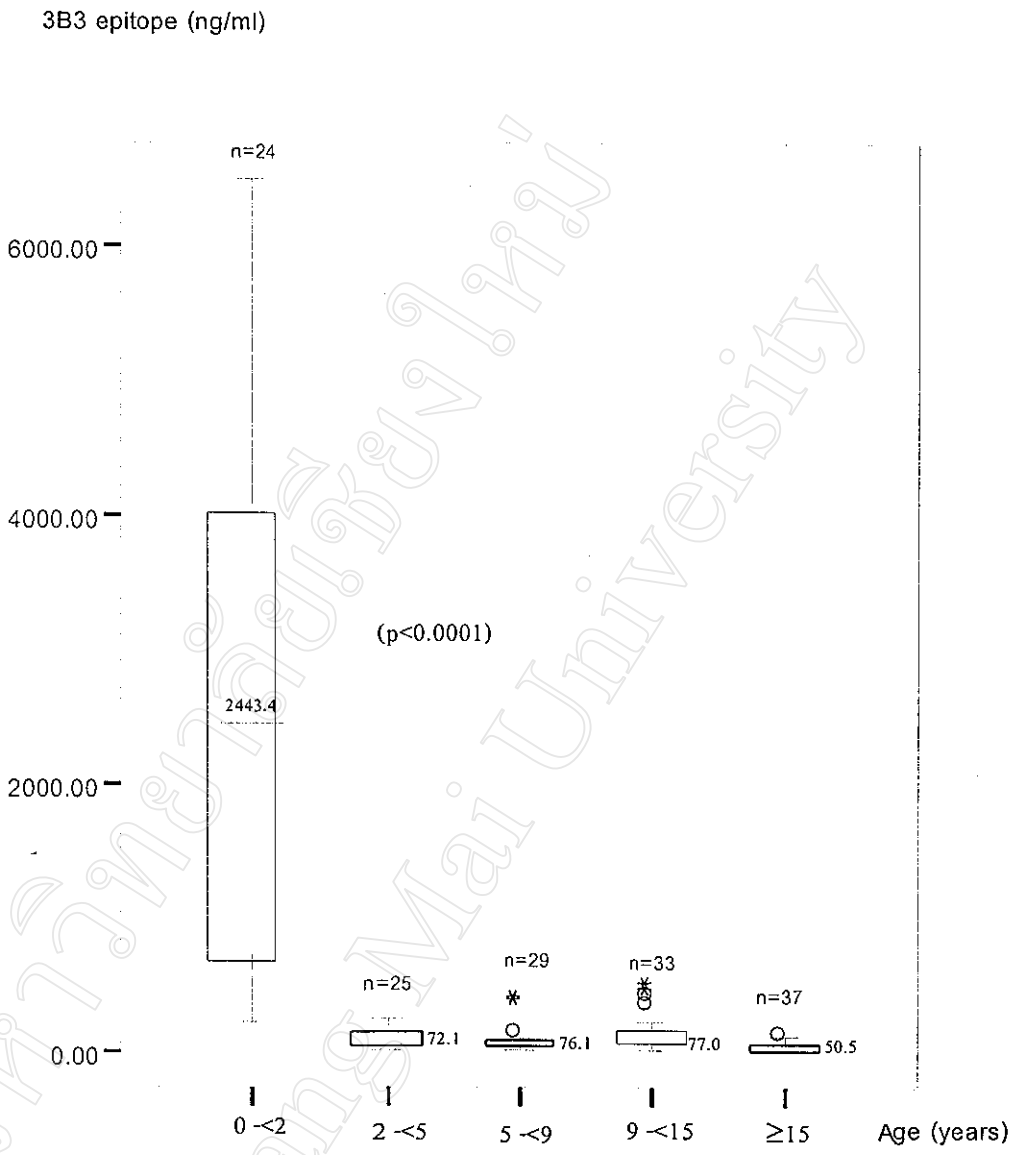

Figure 15 Comparison of serum 3B3 epitope concentration among normal horses at five age groups. Lines in the boxes show median value. Between the upper edge and the lower edge of the boxes showed value at percentile 25-75. And between the line outside the boxes show the maximum and minimum value. Spots show outier values and star show extreme values that were excluded in median calculation. Newborn to less than two-year-old horse had significantly higher 3B3 epitope concentration when compare with other age groups ($p<0.0001$).

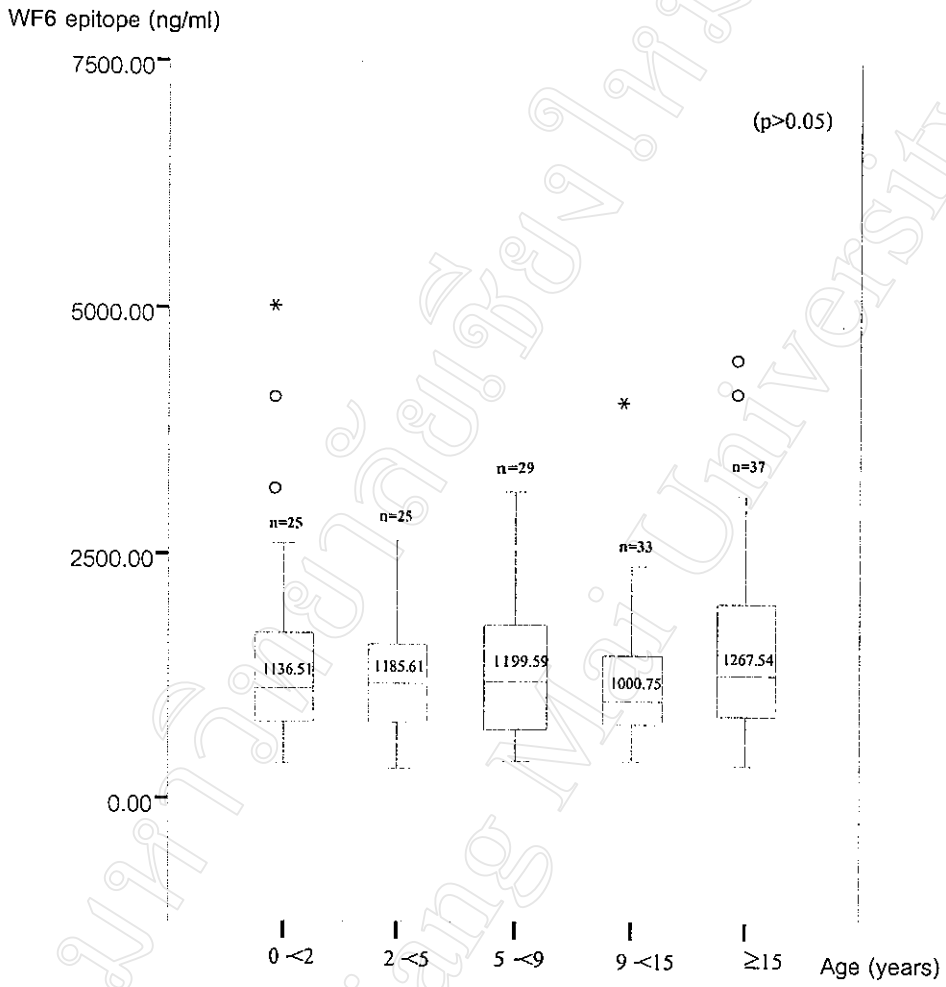

Figure 16 Comparison of serum WF6 epitope concentration among normal horses at five age groups. Lines in the boxes show median value. Between the upper edge and the lower edge of the boxes showed value at percentile 25-75. And between the line outside the boxes show the maximum and minimum value. Spots show outier values and star show extreme values that were excluded in median calculation. There was no different significance of WF6 concentration in any age groups of horses ($p>0.05$).

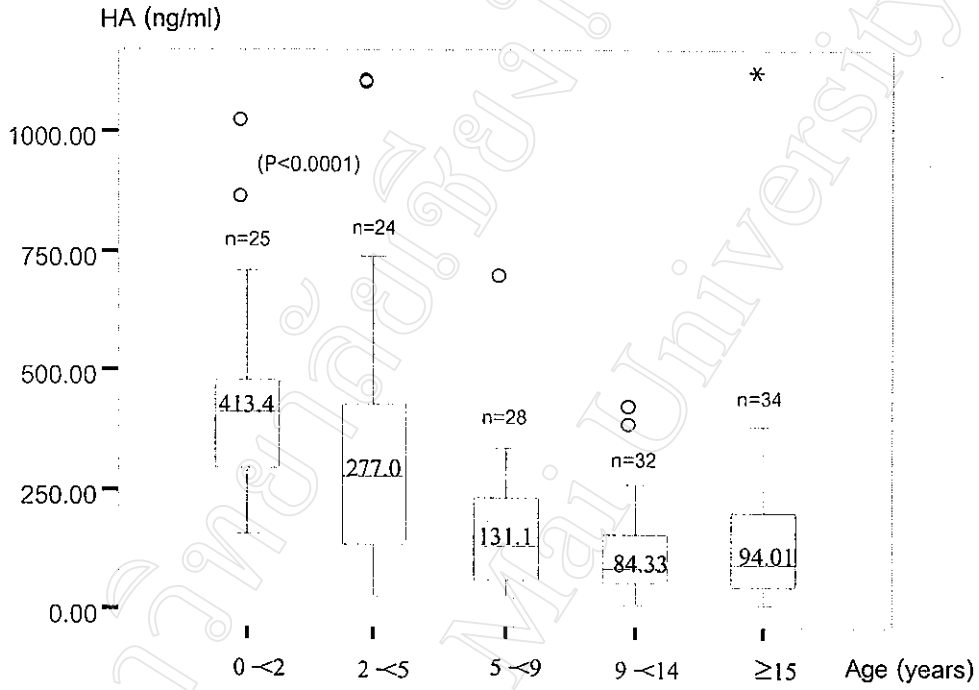

Figure 17 Comparison of serum HA concentration among normal horses at five age groups. Lines in the boxes show median value. Between the upper edge and the lower edge of the boxes showed value at percentile 25-75. And between the line outside the boxes show the maximum and minimum value. Spots show outier values and star show extreme values that were excluded in median calculation. Horse at newborn to less than five years old had significantly higher HA concentration than horses at more than five years old ($p<0.0001$).

Table 12 The level of 3B3 epitope, WF6 epitope, and HA in normal horses categorized by age

Group	3B3 epitopes (ng/ml)	WF6 epitopes (ng/ml)	HA (ng/ml)
Newborn - <2 y	2711.27 ± 1997.89	1500.15 ± 1122.15	435.11 ± 202.44
2 y - <5 y	98.87 ± 66.72	1252.84 ± 628.07	352.49 ± 287.43
5 y - <9 y	107.07 ± 107.21	1319.38 ± 750.21	167.81 ± 138.62
9 y - <15 y	138.02 ± 132.82	1233.58 ± 725.56	116.54 ± 95.06
≥ 15 y	53.97 ± 40.10	2496.80 ± 6984.85	155.79 ± 197.37

Study B: comparison the ECM markers between non-OA horses and OA horse

This study compared the concentration of chondroitin sulfate epitopes (3B3 and WF6 epitope) and HA in non-OA and OA horses.

Table 13 showed mean and standard deviation and also the different significance of these ECM markers in both groups of horses. The median value was shown in Figure 17, 18, and 19. In 3B3 epitope determination, it was found that this marker was significantly lower ($p<0.001$) in OA horses when compared with non-OA horses. Analysis of WF6 epitope showed the contrast result with 3B3 epitope. On the contrary, there was significantly higher ($p<0.0001$) WF6 epitope in OA group when compare with non-OA group. From HA analysis, it was found that there was no different significance ($p>0.05$) between OA and non-OA horses.

Table 13 The level of 3B3 epitope, WF6 epitope, and HA in serum of non-OA and OA horses

Group	3B3 epitope (ng/ml)	WF6 epitope (ng/ml)	HA (ng/ml)
Non-OA	103.28 ± 90.02	1288.57 ± 690.72	253.05 ± 236.80
OA	35.69 ± 36.62	2302.32 ± 1026.78	237.76 ± 278.00
Significance	S	S	NS

NS = non significance ($p>0.05$)

S = significance ($p<0.05$)

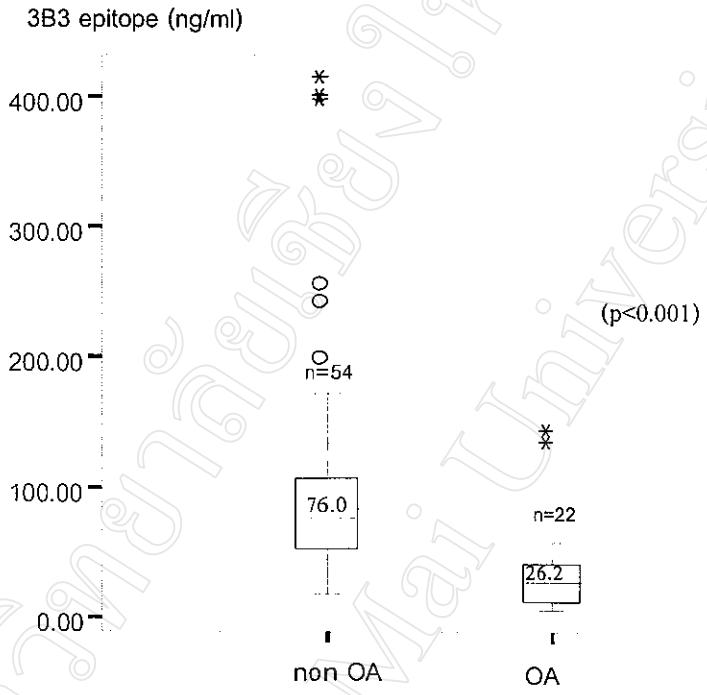

Figure 18 Comparison of serum 3B3 epitope concentration between osteoarthritic horses (OA) and non-osteoarthritic horses (non OA). Lines in the boxes show median value. Between the upper edge and the lower edge of the boxes show value at percentile 25-75. And between the lines outside the boxes show the maximum and minimum values. Spots show outier values and stars show the extreme values that were excluded in median calculation. There was different significance between both of groups ($p < 0.001$)

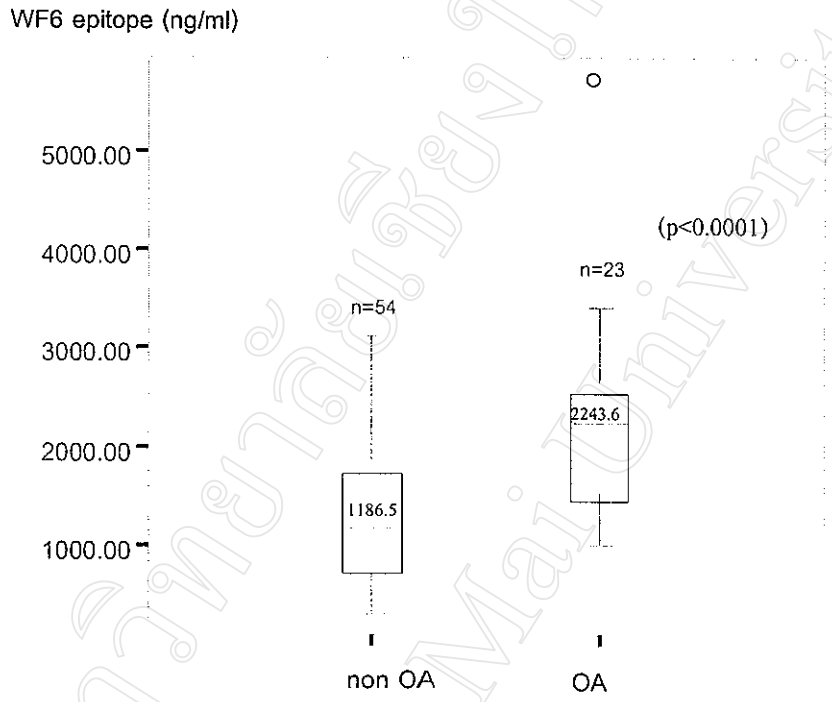

Figure 19 Comparison of serum WF6 epitope concentration between osteoarthritic horses (OA) and non-osteoarthritic horses (non OA). Lines in the boxes show median value. Between the upper edge and the lower edge of the boxes show value at percentile 25-75. And between the lines outside the boxes show the maximum and minimum values. Spots show outier values and stars show the extreme values that were excluded in median calculation. There was different significance in WF6 concentration between both of groups ($p<0.0001$).

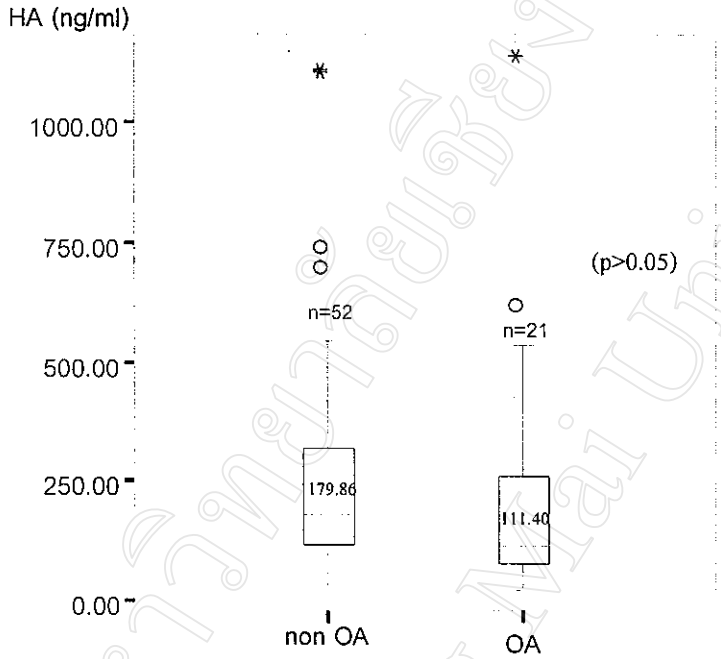

Figure 20 Comparison of serum HA concentration between osteoarthritic horses (OA) and non-osteoarthritic horses (non OA). Lines in the boxes show median value. Between the upper edge and the lower edge of the boxes show value at percentile 25-75. And between the lines outside the boxes show the maximum and minimum values. Spots show outier values and stars show the extreme values that were excluded in median calculation. There was no different significance between both of groups (p>0.05).