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COMPARISON BETWEEN CANINE HEARTWORM AG 2.0 (BIONOTE, KOREA) AND OTHER COMMERCIAL TEST KITS FOR RAPID IN-CLINIC USE.

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Referred to the Study Protocol (Appendix A) of the Agreement between Company OR SELL Srl and the University of Padua, Dep. MAPS

The Study was performed from May to October 2012, with the aim to provide scientific evidences on the quality performances of CANINE HEARTWORM AG 2.0 (CHW AG 2.0) test kit, using blood samples collected on dog populations of both *Dirofilaria immitis* endemic and free areas of North-eastern Italy (see Study Protocol-Appendix A).

1. SAMPLING AND DIAGNOSTIC ACTIVITIES

Blood samplings and diagnostic activities (Table 1) were performed from May to October, 2012 on dogs with different epidemiological history:

- **Group G1**: dogs born and always lived in *D. immitis* free areas, negative for previous assessments to detect circulating *D. immitis* microfilariae (mfe) and antigens (Ags);
- **Group G2**: microfilaraemic dogs, 2-5 years old, living in *D. immitis* endemic areas, and never treated with micro- and/or macro-filaricidal drugs.
- **Group G3**: dogs older than 2 years, and randomly chosen among subjects living in *D. immitis* endemic areas.

Strong efforts have been engaged in endemic areas to provide dogs with qualifications that would allow to include subjects in the G2 group. Out of 17 samples (Table 1), 11 were preserved (frozen) whole blood (n.=4) or sera (n.=7) samples obtained from microfilaraemic dogs previously detected and living in endemic areas (courtesy of Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padua, Italy). So, it was not possible to perform Knott's test on these samples, while PCR analyses were carried out on the 4 whole blood samples.

Table 1. Blood samplings and diagnostic activities performed in the study

Activities	Performed (n.)	Planned (n.) in the Study Protocol
Blood samplings		
Group G1	33	30
Group G2	17	30
Group G3	90	40
Total	140	100
Modified Knott's tests	118	100
Biomolecular analyses	120	100
Serology by CHW AG 2.0	138	100
Serology by SNAP PF	140	100
Serology by WITNESS	138	100

2. DATA ANALYSES

2.1 Concordance calculation

Concordance between different tests has been evaluated by pairwise comparison using k statistic, and choosing a 95% confidence interval (CI). Values of k strength of agreement were interpreted as follows (Altman DG, 1991. Practical statistics for medical research, London, Chapman and Hall):

Value of k	Strength of agreement
<0.200	Poor
0.210-0.400	Fair
0.410-0.600	Moderate
0.610-0.800	Good
0.810-1.000	Very good

The k statistic has been applied to compare the concordance between:

Serological tests:

- CHW AG 2.0 *vs.* SNAP HTWM
- CHW AG 2.0 *vs.*WITNESS
- SNAP HTWM *vs.*WITNESS

Serological tests and modified Knott's tests:

- CHW AG 2.0 *vs.* modified Knott's test
- SNAP HTWM *vs.* modified Knott's test
- WITNESS and *vs.* modified Knott's test

Serological tests and PCR analyses:

- CHW AG 2.0 *vs.* PCR analyses
- SNAP HTWM *vs.* PCR analyses
- WITNESS *vs.* PCR analyses

PCR analyses vs. modified Knott's test.

The software used was SPSS for Windows, version 16.0.

2.2 Calculation of test performances

Performances of each test have been expressed by the calculation of Accuracy (AC), Sensitivity (SE), Specificity (SP), Positive (PPV) and Negative Predictive Values (NPV).
The software used was Win Episcope 2.0, choosing a 95% confidence interval.

2.2.1 Performances of serological tests

Performance indexes (AC, SE, SP, PPV, NPV) have been calculated for each serological test, referring to the level of agreement between the test results and the "true" clinical state of animals, considering the dataset (n=50) including groups G1 (33 uninfected dogs=true negatives) and G2 (17 infected dogs=true positives).

2.2.2 Performances of modified Knott's test

Performance indexes (AC, SE, SP, PPV, NPV) have been calculated for the modified Knott's test, referring its level of agreement with two different datasets:

- the dataset (n=39) including blood samples collected on animals belonging to groups G1 (33 uninfected dogs=true negatives) and G2 (6 infected dogs=true positives). Eleven samples were excluded from group G2 since represented by preserved blood samples, useless to perform the Knott's test.
- the dataset (n=116) including blood samples analysed by both modified Knott's test and PCR, considering this latter as the gold standard for the detection of circulating mfe.

2.2.3 PCR analyses

Samples have been subjected to DNA extraction using the NucleoSpin® Tissue (Macherey-Nagel, Germany), while PCR amplifications of the 5S ribosomal spacer were carried out by S2-S16 primers. PCR products have been purified using the High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim,Germany) and sequenced on both strands at the BMRGenomics of Padua. Sequencing reactions have been analysed, and compared with those available in GenBank using BLAST.

3. RESULTS

3.1 Concordance between serological tests

3.1.1 CHW AG 2.0 vs. SNAP HTWM

		CHW AG 2.0		
		Neg.	Pos.	Total
SNAP HTWM	Neg.	109 100%	0 0%	109 100%
	Pos.	1 3.4%	28 96.6%	29 100%
Total		110 79.7%	28 20.3%	138 100%

k=0.978 - corresponding to a very good agreement.

3.1.2 CHW AG 2.0 vs. WITNESS

		CHW AG 2.0		
		Neg.	Pos.	Total
WITNESS	Neg.	110	7	117
		94.0%	6.0%	100%
	Pos.	0	21	21
		0%	100%	100%
Total		Total	28	138
		79.7%	20.3%	100%

k=0.827 - corresponding to a very good agreement.

3.1.3 SNAP HTWM vs. WITNESS

		WITNESS		
		Neg.	Pos.	Total
SNAP HTWM	Neg.	109	0	109
		100%	0%	100%
	Pos.	8	21	29
		27.6%	72.4%	100%
Total		117	21	138
		84.8%	15.2%	100%

k=0.806 - corresponding to a good agreement.

3.2 Concordance between serological tests and microfilaraemic/amicrofilaraemic dog-status detected by modified Knott's tests

3.2.1 CHW AG 2.0 vs. modified Knott's test

		CHW AG 2.0		
		Neg.	Pos.	Total
KNOTT	Neg.	102	5	107
		95.3%	4.7%	100%
	Pos.	1	8	9
		11.1%	88.9%	100%
Total		103	13	116
		88.8%	11.2%	100%

k=0.700 - corresponding to a good agreement.

3.2.2 SNAP HTWM vs. modified Knott's test

		SNAP HTWM		
		Neg.	Pos.	Total
KNOTT	Neg.	104	5	109
		95.4%	4.6%	100%
	Pos.	0	9	9
		0%	100%	100%
Total		104	14	118
		88.1%	11.9%	100%

k=0.760 - corresponding to a good agreement.

3.2.3 WITNESS vs. modified Knott's test

		WITNESS		
		Neg.	Pos.	Total
KNOTT	Neg.	104	3	107
		97.2%	2.8%	100%
	Pos.	5	4	9
		55.6%	44.4%	100%
Total		109	7	116
		94.0%	6.0%	100%

k=0.464 - corresponding to a moderate agreement.

3.3 Concordance between serological tests and microfilaraemic/amicrofilaraemic dog-status detected by PCR analyses

3.3.1 CHW AG 2.0 vs. PCR analyses

		CHW AG 2.0		
		Neg.	Pos.	Total
PCR	Neg.	98	6	104
		94.2%	5.8%	100%
	Pos.	3	11	14
		21.4%	78.6%	100%
Total		101	17	118
		85.6%	14.4%	100%

k=0.666 - corresponding to a good agreement.

3.3.2 SNAP HTWM vs. PCR analyses

		SNAP HTWM		
		Neg.	Pos.	Total
PCR	Neg.	100	6	106
		94.3%	5.7%	100%
	Pos.	2	12	14
		14.3%	85.7%	100%
Total		102	18	120
		85.0%	15.0%	100%

k=0.712 - corresponding to a good agreement.

3.3.3 WITNESS vs. PCR analyses

		WITNESS		
		Neg.	Pos.	Total
PCR	Neg.	101	3	104
		97.1%	2.9%	100%
	Pos.	7	7	14
		50.0%	50.0%	100%
Total		108	10	118
		91.5%	8.5%	100%

k=0.538 - corresponding to a moderate agreement.

3.4 Concordance between microfilaraemic/amicrofilaraemic dog-status detected by modified Knott's tests and PCR analyses

		PCR		
		Neg.	Pos.	Total
KNOTT	Neg.	104	3	107
		97.2%	2.8%	100%
	Pos.	0	9	9
		0%	100%	100%
Total		104	12	116
		89.7%	10.3%	100%

k=0.843 - corresponding to a very good agreement.

3.5 Performances of serological tests

3.5.1 CHW AG 2.0

		CHW		Total
		Neg.	Pos.	
INFECTED	No	33	0	33
		100%	0%	100%
	Yes	0	17	17
		0%	100%	100%
Total		33	17	50
		66.0%	34.0%	100%

AC, SE, SP, PPV, NPV= 100%

3.5.2 SNAP HTWM

		SNAP HTWM		Total
		Neg.	Pos.	
INFECTED	No	33	0	33
		100%	0%	100%
	Yes	0	17	17
		0%	100%	100%
Total		33	17	50
		66.0%	34.0%	100%

AC, SE, SP, PPV, NPV= 100%

3.5.3 WITNESS

		WITNESS		Total
		Neg.	Pos.	
INFECTED	No	33	0	33
		100%	0%	100%
	Yes	4	13	17
		23.5%	76.5%	100%
Total		37	13	50
		74.0%	26.0%	100%

AC=92.0%; SE=76.5%; SP=100%; PPV=100%; NPV=89.2%

3.6 Performances of modified Knott's test

3.6.1 Referred to true infected/uninfected animals

		KNOTT		Total
		Neg.	Pos.	
INFECTED	No	33	0	33
		100%	0%	100%
	Yes	0	6	6
		0%	100%	100%
Total		33	6	39
		84.6%	15.4%	100%

AC, SE, SP, PPV, NPV= 100%

3.6.2 Referred to PCR results

Considering the level of agreement between modified Knott's test and PCR (see section 3.4), the following performance indexes have been calculated:

AC=74.4%; SE=75.0%; SP=100%; PPV=100%; NPV=97.2%

3.7 PCR analyses

A total of 120 blood samples were tested by PCR analyses. Results are reported in Table 2, referring to the different sample groups (G1, G2 and G3).

Table 2. PCR results referred to the different sample groups

Groups	Tested (n.)	Positive (n.)	Sequencing
G1	32(*)	0	-
G2	10(**)	8	*Dirofilaria immitis*
G3	78	6	*Dirofilaria immitis*
Total	120	14	

(*) One sample was not tested due to the scarcity of blood
(**) 4/10 were frozen whole blood samples (see section 1)

No PCR-positive samples were detected in the negative control group (G1). The presence of *D. immitis* DNA was detected in 8/10 samples belonging to positive control group (G2; Accession numbers: EU 360961.1, EU 360965.1), meanwhile 2 frozen whole blood samples resulted negatives to PCR analyses. Out of 78 samples of G3 group, 6 were positives for *D. immitis* (Accession numbers: EU360964.1, EU 360965.1).

4. COMMENTS

4.1 Samplings

The high difficulty to find blood samples from positive animals (to include in G2 group) was probably due to the large diffusion of prophylactic programs performed by dog owners in endemic areas of the North-eastern Italy. For this reason, it was possible to include in the study only 17 samples, 6 collected directly on animals and 11 given by Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Padua, Italy), of which 4 whole blood and 7 sera, obtained from microfilaraemic dogs previously detected and living in endemic areas.

4.2 Comparison of serological test performances

Very good strengths of agreement have been detected between CHW AG 2.0 and the most used commercial kits SNAP HTWM (k=0.978) and WITNESS (k=0.827) (see section 3.1).
Only one blood sample (in group G3) resulted negative to both immunochromatographic tests CHW AG 2.0 and WITNESS, giving positive result to the ELISA SNAP HTWM. In this sample, circulating mfe were detected by modified Knott's test and PCR analysis.
Both CHW AG 2.0 and SNAP HTWM detected as positive 7 samples that, on the contrary, gave negative results by WITNESS test. Among these samples, 4 belonged to the group G2 (positive controls), and 3 to the group G3 (randomly collected on subjects living in *D. immitis* endemic areas).
Although a very good strength of agreement has been observed between serological tests in the pairwise comparisons performed by k statistic, it is worthy of note that both CHW AG 2.0 and SNAP HTWM showed all performance index values (AC, SE, SP, PPV, NPV) corresponding to 100%, whereas lower values of SE (76.5%; CI=56.3-96.6) and NPV (89.2%; CI=79.2-99.2) have been detected for WITNESS test (see section 3.5). Compare to this latter, CHW AG 2.0 seems to be the immunochromatographic test showing better performances for the rapid detection of HW infections in clinical practice, reducing the diagnostic risk of false negative samples.

4.3 Detection of occult heartworm (HW) infections

A total of 116 blood samples were analysed by Knott's test and all serological tests chosen for the study. Among these samples, 9 (7.8%) were positive to modified Knott's test, 14 (12.1%) to SNAP HTWM, 13 (11.2%) to CHW AG 2.0, and only 7 (6.0%) to WITNESS, confirming this latter as the test with the lower sensitivity.
A total of 5/107 (4.7%) cases of occult HW infections (i.e., positive to at least one of the serological tests and negative to Knott's test) were detected, all belonging to dogs of the Group G3. All these cases were detected both by CHW AG 2.0 and SNAP HTWM test kits, while WITNESS test kit allowed to detect 3/107 (2.8%) cases of occult HW infection.

4.4 Concordance between serological tests and microfilariae detection

Both for SNAP HTWM and CHW AG 2.0, the analyses performed by k statistic showed a good strength of agreement (0.666≤k≤0.760) with modified Knott's test and PCR (see sections 3.2 and 3.3), meanwhile only a moderate strength of agreement was observed in the pairwise comparison WITNESS *vs.* modified Knott's test (k=0.464) and WITNESS *vs.* PCR analyses (k=0.538). Nevertheless, it is important to highlight that serological tests and mfe detections represent diagnostic approaches strongly different in their "target" (goals in *D. immitis* antigens and embryos detection, respectively). So, a disagreement between these diagnostic approaches is clearly expected, and may be due to: *(a)* the possibility of occult HW infections (see section 4.3), and/or *(b)* a very low level of circulating antigens (Ags) as consequence of adult death, meanwhile circulating mfe are still present as consequence of their ability to survive for longer time (till to one year or more) than Ags. In this study, both *(a)* and *(b)* hypotheses must be excluded for animals enrolled in G1 and G2 groups, according to the conditions defined by the protocol (negative and positive controls, respectively; see section 1). On these basis, it is worthy of note that all samples negatives to CHW AG 2.0 and SNAP HTWM, but positives to modified Knott's test (1 and 0 samples, respectively; see section *3.2.1* and *3.2.2*) and PCR (3 and 2 samples, respectively; see section *3.3.1* and *3.3.2*), belonged to the group G3 (including blood samples randomly collected on dogs living in *D. immitis* endemic areas). On the contrary, disagreements between WITNESS negativity and circulating mfe detection were observed also in 3 samples belonging to the group G2 (positive controls). In particular, circulating microfilariae were detected in 5 (3 of G2, and 2 of G3) and 7 (3 of G2, and 4 in G3) WITNESS negative samples by modified Knott's test and PCR analyses, respectively (see sections *3.2.3* and *3.3.3*).

4.5 Performances of modified Knott's test

Results on modified Knott's test performances (see section 3.6) need a very careful evaluation. The high value (100%) of performance indexes calculated for this test lacks in the low number of positive controls (true positive=6) that has been possible to include in G2 group, and in the fact that a microfilaraemic condition were required to enrol animals in the same group of sampling. For this reasons, and to avoid misunderstanding on the evaluations concerning modified Knott's test performances, it has been considered appropriate to apply k statistic as pairwise comparison between modified Knott's test and PCR (see sections 3.4 and *3.6.2*), considering this latter as the gold standard (more sensitive test) for the detection of circulating mfe. Though a very good strength of agreement (k=0.843) has been detected in this comparison, modified Knott's test show the ability to perform a correct diagnosis (AC=Accuracy value) in the 74.4% of the cases, with a sensibility (SE=proportion of infected animals detected by the test) of 75% (CI=50.5%-99.5%) and NPV (NPV=probability that animals tested as negative are really uninfected) of 97.2% (94.1%-100%). Otherwise, modified Knott's test show a value of 100% in SP (specificity) and PPV (Positive Predictive Value), corresponding to the probability that animals tested as positive are really infected.

Moreover, it is important to highlight that modified Knott's test, as any other diagnostic technics (including PCR analyses) having the goal to detect circulating mfe, may fails in diagnosis giving false negative results in the case of occult HW infections, also due in North-eastern Italy to an inappropriate *off label* use of ivermectin during HW prophylactic programmes. This is also evidenced by the cases of occult HW infections detected in this study (see section 4.3).

5. CONCLUSIONS

- **CHW AG 2.0** performances seems to be equivalent to that of the SNAP HTWM, a test largely used in Italy by veterinary practitioner.
- Compare to the immunochromatographic WITNESS test, **CHW AG 2.0** show better performances for the rapid detection of HW infections in clinical practice, reducing the diagnostic risk of false negative samples.
- Compare to SNAP HTWM and WITNESS, **CHW AG 2.0** is more rapid and easier to use during clinical activities (fewer steps are required to the veterinarian)

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