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RESEARCH ARTICLE

DEVELOPMENT AND ASSESSMENT OF A COAGGLUTINATION TEST FOR DETECTING CANINE PARVOVIRUS IN CLINICAL SPECIMENS.

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Abstract

Rapid diagnosis of canine parvovirus (CPV-2) infection is of key importance in management and control of the disease. The present study aims at the development and evaluation of an easy to use rapid test for the detection of canine parvovirus particles in clinical samples collected and tested during daily veterinary practice.

For this purpose, the coagglutination test (COA) using Methylene blue stained anti-CPV2 antibodies sensitised *staphylococcus aureus* ATCC 12598 was performed to detect CPV-2 in 91 clinical samples (86 rectal swabs and 5 organ homogenates) collected between 2011 and 2015 from suspected canine clinical cases received in Moroccan veterinary clinics. The same samples were previously tested by Hemagglutination test and real-time PCR (Amrani et al., 2016), the results were compared and employed in the evaluation of COA.

The results of the COA showed 79.12% (72/91) of tested samples to be positive and 20.87% (19/91) to be negative. The intensity of reaction in positive samples was scored high (3+) in 6 samples (6.6%), medium (2+) in 28 samples (30.7%) and low (1+) in 57 samples (62.6%) which occurred within a median time of 3min, 4min and 3min42s, respectively. In comparison with PCR, the COA test demonstrated a good agreement of 80.2 % and a sensitivity of 80%. The coagglutination test is a promising test allowing daily diagnostic of canine parvovirus in field, veterinary clinics and non- equipped laboratories with molecular techniques.

Introduction:-

Digestive disorders including diarrhoea and vomiting are considered as major canine health problems and the most frequent cause for veterinary consultation (Rakha et al., 2015; Ylmaz et al., 2002). Among numerous aetiologies involved in these disorders, canine parvovirus (CPV-2) infection is the most frequent and serious pathology, characterized by high morbidity and mortality rates, especially in susceptible puppies of 6 weeks to 6 months of age (Prittie, 2004). The causative CPV-2 is a non-enveloped virus with icosahedral capsid enclosing a single stranded DNA of ~5000bp characterized by a high mutation rate similar to that observed for RNA viruses (Decaro et al., 2009). Since its emergence, CPV-2 has rapidly evolved giving rise to highly pathogenic variants CPV-2a, 2b and 2c (Decaro and Buonavoglia, 2012). Vaccination remains the main tool to control and prevent canine parvovirus

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infection. However, vaccine neutralisation by long-lasting maternal antibodies (MDA) associated with poor or absence of cross-protection between CPV-2 antigenic variants, compromise seriously the success of vaccination (Decaro et al., 2005a, 2008). Early, rapid, accurate diagnosis and management of CPV-2 is of key importance in effective reduction of infected dog's morbidity and mortality (Kim et al., 2015). Molecular diagnostic tests, notably the real-time PCR, were demonstrated to be more sensitive than traditional methods (Decaro et al., 2005b; Desario et al., 2005). However, PCR-based tests are expensive, carried out in specialized laboratories only and are difficult to establish for standard practice in veterinary clinics. Therefore, development of rapid, simple, sensitive and affordable tests detecting CPV-2 particles in clinical samples would be convenient and can be conducted in veterinary clinical practice (Kantere et al., 2015). The coagglutination (COA) test was previously developed (Genovn and Ivanov, 2006; Singh et al., 1998) by exploiting the property of *staphylococcus aureus* membrane protein A to bind the Fc portion of anti-parvovirus IgG which recognizes CPV-2 antigen in positive tested samples. Compared with Haemagglutination test (HA), this test was reported specific and sensitive (Genovn and Ivanov, 2006).

The aim of the present study is to develop an easy to use and interpret coagglutination assay and to evaluate its performances (sensitivity, positive predictive value, overall agreement) in comparison with real-time PCR as gold standard test for canine parvovirus detection.

Material and Methods:-

Clinical samples:-

A total of 91 clinical samples consisting of 86 rectal swabs and 5 organ's homogenates were collected from suspected dogs suffering from diarrhoea and vomiting, received in veterinary clinics located in Marrakech, Settat, Khemisset, Casablanca, Temara, Rabat, Sale and Machraa Belakssiri, between 2011 and 2015. These samples were previously processed and screened for canine parvovirus infection by means of real-time PCR and HA (Amrani et al., 2016) (table1).

Bacteria:-

Lyophilised *Staphylococcus aureus* ATCC 12598 was reconstituted and revived following the manufacturer's recommendations. An isolated *S. aureus* colony was cultured into Brain and Heart broth (BHB) for 18h at 37°C in a shaking water bath. The resulting bacterial culture was washed 3 times in PBS and inactivated by adding 0.5% formaldehyde solution at 4°C for 18h and washed and heated at 80°C for 20min. Following inactivation, bacteria were washed three times and resuspended in PBS containing 0.5% Tween 20. Using spectrophotometry at 250nm, bacterial absorbance was fixed at 0.69, corresponding to 2.3 x 10^{10}cells/ml. This suspension was stored at 4°C for maximum 3 months (Montassier et al., 1994).

Production of hyper immune serum:-

As shown in figure 1, Anti-parvovirus hyperimmune serum was produced on SPF, New Zealand, male rabbit weighting 3.5kg. Briefly, three CPV-2 vaccine doses (Primodog®, CPV strain C-780916 $\geq 10^{5.5}$ $DICC_{50}$) were inoculated by subcutaneous route on day 0, 15 and 30, respectively. At day 40, blood was collected from the hyperimmunized rabbit and serum was separated by centrifugation. After decomplementation at 56°C for 30 min, hyperimmune serum was mixed with the previously described *S aureus* suspension at 2:1 (V/V) and incubated for 3h at room temperature. To facilitate the test lecture, IgG−*S.Aureus* complexes were stained using 0.075% methylen blue stain. The produced reagent was stored at 4°C for use during one month.

Test procedure:-

The coagglutination test was performed using a glass slide by homogenising equal volumes (50 µl) of clinical samples supernatants with stained IgG−*S. Aureus* reagent according to the method described previously (Genovn and Ivanov, 2006). The slide was examined macroscopically during 10 min for development of agglutinin particles. The time and the intensity of the reaction (1+, 2+, 3+) were recorded (figure 2). Positive and negative controls consisting of a positive and negative real-time PCR fecal samples collected from dogs were used. The overall procedure is summarised in figure 1.

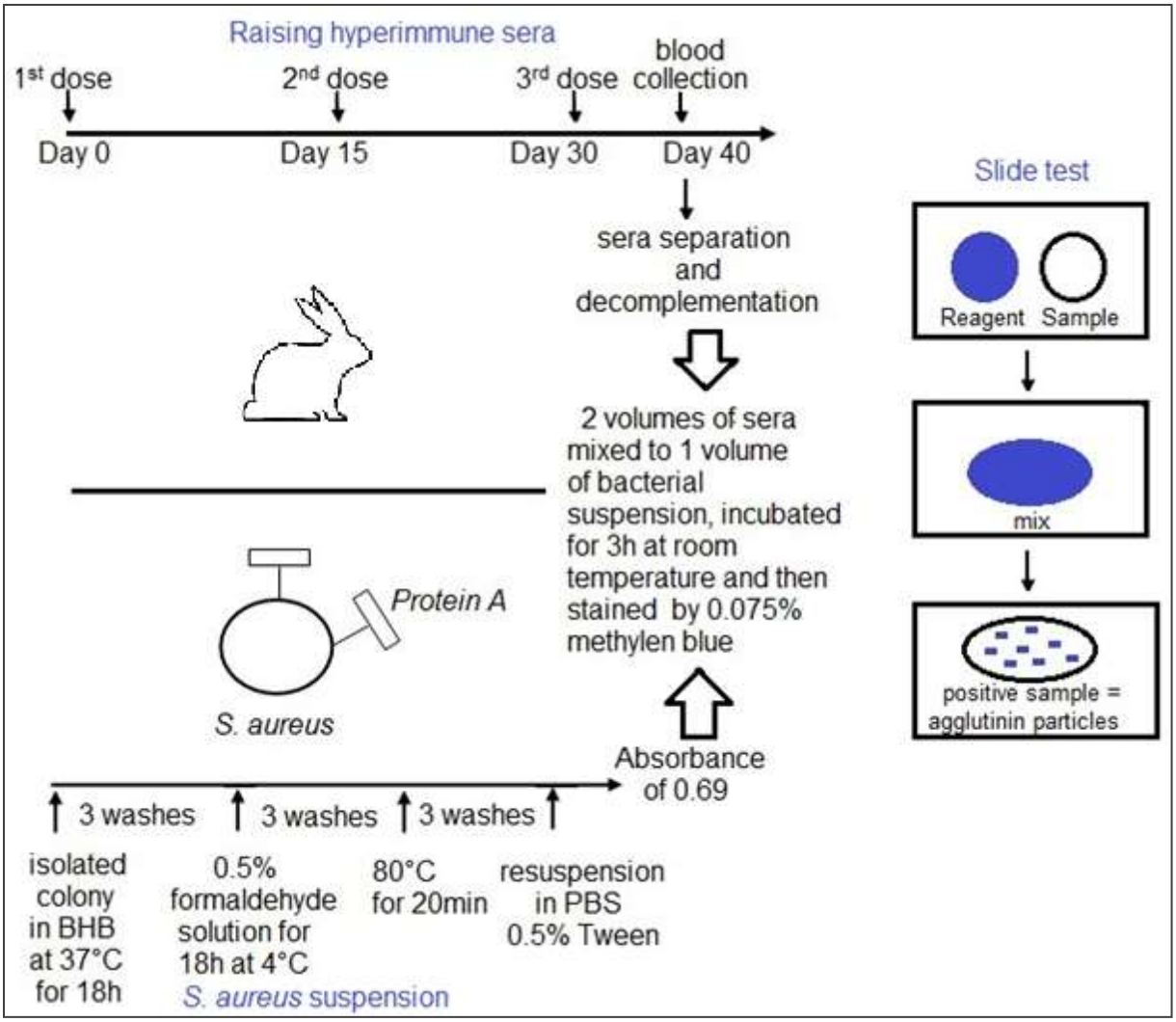

Figure 1:-Reagent preparation and specimen testing protocol

The test performances were evaluated by calculating sensitivity and overall agreement in comparison with the gold standard test, real-time PCR, carried out previously for the same samples (Amrani et al., 2016).

Results:-

Obtained results and information about tested samples are presented in table 1.

Among 91 tested samples, the coagglutination test detected 72 positive samples (79.12%) and 19 negative samples (20.87%). Agglutinin particles appeared within 45s to 10min after mixing the reagent with samples (figure2). The intensity of reaction and time of its appearance was scored from 1+ to 3+ and in seconds, respectively. Agglutinin particles concentration was high (3+) in 6 samples (6.6%), median (2+) in 28 samples (30.7%) and low (1+) in 57 samples (62.6%).

Positive reactions occurred within a median time of 3min, 4min and 3min42s respectively (figure3). Sensitivity of the test reached 80% (72/90) and overall agreement accounted for 80.2% between COA and PCR tests (73/91).

Table 1:-Informations regarding samples, DNA loads using real-time PCR, scored COA intesity and reaction time

Code	Sample	Year	Age by month	Breed	Sex	Vaccination	Origin	Real-time PCR (DNA copy/gm)	COA intensity	Reaction time
Alg-11	I	2011	NA	NA	NA	NA	Marrakech	1.1×10^5	1+	3min40s
S10-11	I	2011	2	R	F	UV	Rabat	1.7×10^8	1+	3min25s
Dark11	S	2011	NA	NA	NA	NA	NA	2.7×10^7	N	-
Parvo 3	I	2012	1	R	F	UV	Rabat	1.3×10^8	1+	3min
Parvo 4	I	2012	NA	NA	NA	NA	Rabat	3.8×10^6	1+	3min
P01-13	F	2013	3	P	F	V	Rabat	$1,3 \times 10^9$	2+	4min05s
P02-13	F	2013	4	LR	M	V	Rabat	2.8×10^9	N	-
P03-13	I	2013	6	LR	F	V	Rabat	4.6×10^5	1+	3min55s
P04-13	F	2013	6	GR	F	V	Rabat	7.5×10^4	1+	3min05s
P05-13	M,S	2013	3	MB	F	V	Rabat	1.1×10^6	N	-
P06-13	F	2013	2	P	M	UV	Rabat	3.5×10^7	2+	3min58s
P09-13	S	2013	6	MB	NA	UV	Rabat	4.2×10^6	N	-
P1-13	I	2013	4	MB	M	UV	Khemisset	1.6×10^8	1+	3min45s
P2-13	I	2013	3	MB	M	UV	Khemisset	4.6×10^9	1+	3min56s
P3-13	F	2013	3	MB	F	UV	Khemisset	5×10^4	2+	1min36s
P4-13	I	2013	3	MB	F	UV	Khemisset	8.2×10^6	1+	3min50s
P5-13	I	2013	3	MB	M	UV	Khemisset	2.3×10^8	2+	4min25s
P6-13	I	2013	3	MB	M	UV	Khemisset	1.4×10^9	N	-
P7-13	I	2013	3	MB	M	UV	Khemisset	1.7×10^6	2+	3min56s
P8-13	L,S,M	2013	3	MB	F	UV	Khemisset	6.1×10^6	1+	2min40s
P9-13	L	2013	3	MB	F	UV	Khemisset	3.4×10^6	1+	7min17s
P10-13	I	2013	3	MB	F	UV	Khemisset	3.3×10^9	N	-
P11-13	F	2013	3	MB	M	UV	Khemisset	8.6×10^6	N	-
P12-13	F	2013	6	MB	F	UV	Khemisset	2×10^{10}	2+	3min
P13-13	F	2013	5	MB	F	UV	Khemisset	4.2×10^8	N	-
P14-13	F	2013	5	GS	M	V	Rabat	3.4×10^8	N	-
P15-13	F	2013	5	GS	M	V	Rabat	$5,3 \times 10^8$	N	-
P16-13	I	2013	5	St. G	M	UV	Rabat	2.3×10^7	N	-
P5-14	I	2014	6	R	M	V	Rabat	2.4×10^6	2+	5min41s
P6-14	F	2014	4	R	F	UV	Casablanca	1.4×10^8	2+	6min14s
P7-14	F	2014	4	H	M	V	Casablanca	4.7×10^5	1+	6min24s
P8-14	F	2014	4	St. G	F	V	Casablanca	2.7×10^6	N	-
P9-14	I	2014	3	C	F	V	Casablanca	3.3×10^8	1+	7min30s
P10-14	F	2014	2	BF	M	V	Casablanca	$3,4 \times 10^9$	1+	5min05s
P11-14	F	2014	3	H	F	V	Settat	8×10^7	1+	3min56s
P12-14	I	2014	4	C	M	V	Casablanca	1.1×10^5	1+	6min42s
P13-14	I	2014	2	LA	M	V	Casablanca	6.2×10^8	2+	4min56s
P14-14	I	2014	3	GS	M	V	Casablanca	$1,2 \times 10^9$	2+	3min56s
P15-14	F	2014	3	GS	M	V	Casablanca	$2,8 \times 10^6$	2+	5min30s
P16-14	F	2014	3	GS	M	V	Casablanca	1.4×10^8	1+	6min34s
P17-14	F	2014	3	GS	F	V	Casablanca	6.7×10^6	3+	3min50s
P18-14	F	2014	1	MB	M	UV	Rabat	1.9×10^8	2+	5min27s
P1-15	F	2015	7	MB	M	UV	Rabat	2.1×10^6	1+	4min19s
P2-15	F	2015	3	GS	M	V	Rabat	8.4×10^8	3+	4min17s
P3-15	F	2015	5	GS	M	UV	Rabat	9.8×10^4	2+	4min19s
P4-15	F	2015	7	GS	M	V	Rabat	2.1×10^6	1+	4min30s
P5-15	F	2015	8	R	M	V	Rabat	3.1×10^8	1+	4min30s
P6-15	F	2015	6	MB	M	UV	Machraa Belaksiri	8.8×10^4	1+	4min35s

Code	Sample	Year	Age by month	Breed	Sex	Vaccination	Origin	Real-time PCR (DNA copy/gm)	COA intensity	Reaction time
P7-15	F	2015	7	MB	M	UV	Machraa Belaksiri	9.7×10^3	1+	4min40s
P8-15	F	2015	7	L	M	UV	Rabat	2.8×10^5	1+	4min48s
P9-15	F	2015	6	MB	M	UV	Rabat	1×10^8	1+	5min
P10-15	F	2015	3	MB	M	UV	Rabat	7.1×10^7	N	-
P11-15	F	2015	5	GS	M	UV	Rabat	$1,2 \times 10^8$	2+	7min12s
P12-15	F	2015	NA	NA	NA	NA	NA	7.8×10^6	2+	10min
P13-15	F	2015	2	GS	M	V	Rabat	1.5×10^4	1+	8min48s
P14-15	F	2015	3	P	F	UV	Rabat	5.4×10^7	1+	9min10s
P15-15	F	2015	24	ASD	F	V	Rabat	6×10^7	1+	7min17s
P16-15	F	2015	8	BS	F	V	Rabat	5.6×10^7	2+	6min
P17-15	F	2015	4	GS	F	V	Rabat	7.3×10^7	2+	4min28s
P18-15	F	2015	3	GS	M	UV	Rabat	1.2×10^5	3+	1min
PC	F	2015	5	MB	F	UV	Rabat	N	N	-
P20-15	F	2015	5	P	F	V	Rabat	8.5×10^8	2+	5min46s
P21-15	F	2015	1	R	M	V	Rabat	2.5×10^6	3+	4min16s
P22-15	F	2015	4	GS	M	UV	Rabat	1.5×10^8	2+	3min
P23-15	F	2015	NA	NA	NA	NA	Temara	2.3×10^9	1+	3min20s
P24-15	F	2015	4	R	M	V	Rabat	9.4×10^6	N	-
P25-15	F	2015	5	St. G	M	UV	Rabat	$1.6\ \ 10^9$	1+	10min
P26-15	F	2015	5	St. G	M	UV	Rabat	9.8×10^5	1+	6min27s
P27-15	F	2015	5	St. G	M	UV	Rabat	5.9×10^4	3+	45s
P28-15	F	2015	5	St. G	M	UV	Rabat	4.1×10^5	1+	5min26s
P29-15	F	2015	5	MB	M	UV	Rabat	N	2+	3min51s
P30-15	F	2015	2	LR	F	V	Temara	4.3×10^7	2+	4min20s
P31-15	F	2015	2	LR	M	V	Temara	5.4×10^4	1+	7min50s
P32-15	F	2015	3	LR	F	V	Temara	2×10^6	1+	5min50s
P33-15	F	2015	2	LR	M	V	Temara	3.3×10^7	1+	6min51s
P34-15	F	2015	3	MB	M	UV	Rabat	2.6×10^8	2+	6min
P35-15	F	2015	3	MB	M	UV	Rabat	1.3×10^9	2+	6min43s
P36-15	F	2015	4	MB	F	UV	Rabat	1.2×10^6	N	-
P37-15	F	2015	4	LR	M	UV	Rabat	4.2×10^7	2+	7min
P38-15	F	2015	4	MB	M	UV	Rabat	1.1×10^8	1+	7min34s
P39-15	F	2015	2	LR	F	V	Temara	6.7×10^6	1+	8min
P40-15	F	2015	2	St. G	M	V	Temara	2.1×10^6	2+	4min55s
P41-15	F	2015	2	St. G	M	V	Temara	5.4×10^5	N	-
P42-15	F	2015	3	MB	M	UV	Rabat	4.9×10^8	N	-
P43-15	F	2015	3	MB	M	UV	Rabat	6.2×10^7	1+	10min
P44-15	F	2015	2	BS	F	UV	Temara	1.8×10^5	1+	10min
P45-15	F	2015	2	BS	F	UV	Temara	5.2×10^5	2+	3min57s
P46-15	F	2015	2	BS	F	UV	Temara	9.9×10^5	2+	4min52s
P47-15	F	2015	7	MB	M	UV	Rabat	1.7×10^6	N	-
P48-15	F	2015	4	BS	M	UV	Rabat	1.1×10^7	3+	3min58s
PA	F	2015	1	R	F	V	Sale	N	2+	4min27s

M: Male ; F: Female ; I: Intestine ; L: Liver ; S: Spleen ; M: Myocardium ; F: Feces ; V: Vaccinated ; UV: Unvaccinated ; NA: No information available ; ASD: Atlas Shepherd Dog ; BS: Belgian Shepherd ; BF: bichon frise ; C: Chihuahua ; GR: Golden retriever ; GS: German shepherd ; H: Husky ; LA: Lhassa Apso ; LR: Labrador Retriever ; MB: Mixed breed ; P: Poodle ; R: Rottweiler ; St. G: Saint-Germain Pointing Dog ;N: Negative; 1+ : low intensity; 2+: median intensity; 3+: high intensity

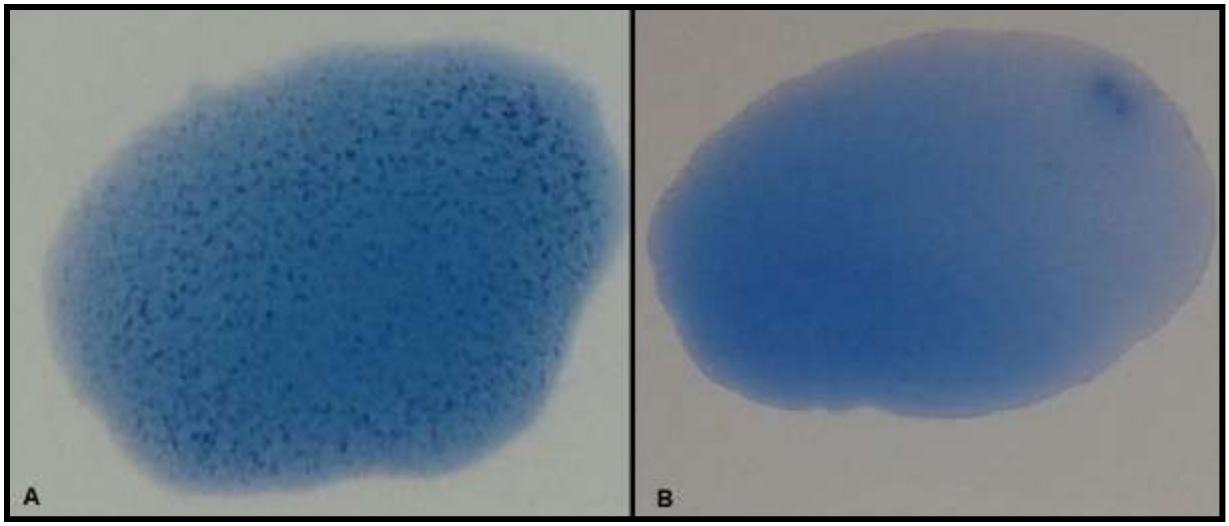

Figure 2:-Coagglutination test after mixing the reagent with positive and negative samples; A: Agglutinin particles in positive reaction; B: negative reaction

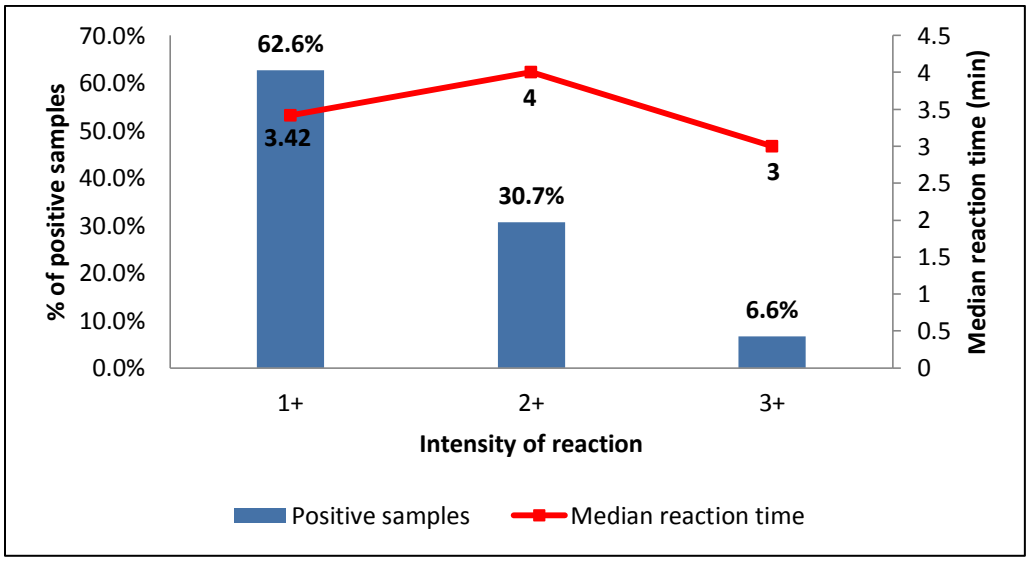

Figure 3:-Intensity of reaction in positive samples and average of the scored reaction time (1+: low reaction, 2+: medium reaction, 3+: High reaction)

Discussion:-

Rapid diagnosis of canine parvovirus remains the first important step to adopt the appropriate measures for disease control and transmission reduction especially in kennels. Molecular based diagnostic methods were demonstrated specific and sensitive (Decaro et al., 2005b; Desario et al., 2005) but cannot be performed in routine veterinary practice as these assays require skill and expensive equipment. The heamagglutination test was described as a reference test in labs that are not equipped with molecular platforms (Amrani et al., 2016; Desario et al., 2005) (Amrani et al., 2016). Nevertheless the difficulty in obtaining fresh pig erythrocytes and the poor correlation with PCR (Desario et al., 2005) hampers the use of this test in routine veterinary practice. Thus, the development of an inexpensive, simple, rapid, specific, sensitive and easy to use diagnostic test in veterinary practice is useful. The coagglutination test was previously performed for CPV-2 (Genovn and Ivanov, 2006) and diverse pathogens detection (Joshi et al., 1989; Montassier et al., 1994; Yoshimizu and Kimura, 1985). We investigated the potential use of COA to detect CPV-2 in clinical samples and evaluated its performances in comparison with the PCR test.

Previous COA assays required dark background or using microscope (x10) to be read macroscopically (Yoshimizu and Kimura, 1985) with the possibility to affect the test lecture and to determine its intensity. Thus, COA using

methylen blue stained reagent provides an easy alternative and more objective lecture without the use of a microscope. A good agreement (80.2%) was found between COA and PCR as a gold standard test. In addition, no false positive samples was observed with COA test compared with haemagglutination test which was shown to be poorly sensitive, resulting in a high proportion of false-negative results. Hence, the COA test developed in the present study can be considered as a good alternative for use as a bench or penside test in clinical veterinary practice.

Summary:-
We report in the current study the development of a, simple, reliable and rapid test to detect canine parvovirus in daily veterinary practice. High performances, processing of many samples, easy procedure and results interpretation make the COA an effective tool especially in field conditions and laboratories that are not equipped with molecular technology platform.

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