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Validation of an improved reference freeze-dried direct agglutination test for detecting leishmaniasis in the canine reservoir

--Manuscript Draft--

Manuscript Title: Validation of an improved reference freeze-dried direct agglutination test for detecting leishmaniasis in the canine reservoir

Running title: Improved FD-DAT for CVL

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ABSTRACT:

Introduction: Proper identification and management of reservoir post-kala-azar dermal leishmaniasis (PKDL) and canine visceral leishmaniasis (CVL) cases are prerequisites to the effective control of visceral leishmaniasis (VL) worldwide. Unlike PKDL, CVL still awaits effective improvement because of its cryptic nature, absence of *Leishmania* parasite in lesions or lymph nodes, and insensitivity of tools in current use. Because of the need for certain skills and equipment, both the liquid direct agglutination test (LQ-DAT) and freeze-dried direct agglutination test (FD-DAT) versions, present in comparison with the indirect immunofluorescence (IFAT) or enzyme-linked immunosorbent assay (ELISA), practical and feasible diagnostic alternatives.

Aim: Validate the performance of an improved FD-DAT to suit routine and large-scale applications in CVL endemic areas.

Methodology: Introducing of citrate-saline formaldehyde (CSF) as an anti-clumping agent to replace normal saline for antigen reconstitution and drastically however eligibly lowering the concentration of promastigotes ($1.4X10^7$) in comparison with the original reference FD-DAT ($\geq 5X10^7$/ml), To ensure optimal safety, β-mercaptoethanol (β-ME) was replaced by urea or sodium dodecyl sulphate (SDS) as a serum reducing agent.

Results: Through improving the procedure for reconstitution of FD-DAT antigen with CSF a 150% reduction in the test application cost was achieved. Expired test batches (± 4 years earlier), were successfully revitalized to full validity. As compared to 48-hour shelf-life time for the original, an FD-DAT batch re-constituted here with CSF maintained validity for ± 12 months.

3

INTRODUCTION:

Because of assimilation with other various canine disorders, scarcity or complete absence of the causative *Leishmania* parasite in the lesions formed, confirmation of canine visceral leishmaniasis (CVL) still constitutes a major draw-back for visceral leishmaniasis (VL) control in South America and North Africa. Although lymph node aspiration is routinely performed in endemic areas, failure to demonstrate *Leishmania* amastigotes in genuine CVL cases was frequently reported [1]. The indirect immunofluorescence antibody test (IFAT), even though considered complicated to perform, is applied at the central laboratory level in the endemic areas of Southern and Eastern Europe [2]. Depending on the nature of the antigen used, variable reliability levels for CVL diagnosis were reported for the Enzyme-linked immunosorbent assay (ELISA) [3-5].

Employing antigen suspension of trypsin-treated and Coomassie Brilliant Blue-stained *L. donovani* promastigotes in a liquid direct agglutination test (LQ-DAT), highly favorable results were reported for VL diagnosis in East Africa [6,7]. By applying the same antigen to sera from Dutch and German dog populations that returned from winter stays with their owners in Southern Europe, diagnostic reliabilities for CVL highly comparable to those of the Eastern African VL suspects were reported [8]. In order to achieve better stability during transportation or storage under adverse high temperatures, a freeze-dried version (FD-DAT) of the test was developed and commercialized in the Netherlands [9,10]. The outcome of evaluation using this improved DAT revealed highly encouraging reliabilities for CVL in Dutch dogs that had overwintered with owners in Southern Europe [9]. However, despite the excellent detection reliability reported, routine application of the FD-DAT was not implemented in the CVL major endemic areas highly likely due to test infrequent availability,

failure to produce the test locally, or the high importation cost involved. The health and environmental hazards associated with mandatory use of β-mercaptoethanol (β-ME) as serum reducing agent in test procedure furthermore formed additional obstacle [11].

During the past decade, significant progress was made that included the production of the LQ-DAT locally and the introduction of essential improvements to the FD-DAT to ensure optimal safety for VL routine diagnosis in Sudan and elsewhere. Aside from a pronounced reduction in test cost using the valid batches, FD-DAT batches that had expired seven years earlier were successfully revitalized contributing to a further lowering the test expenses [12].

In this study, we intended to assess the performances of an improved valid or expired FD-DAT version in comparison with the original reference, a liquid test version (LQ-DAT), IFAT, and an enzyme-linked immunosorbent assay (ELISA) version employing a recombinant antigen for the detection of CVL in an endemic dog population from Croatia.

Methods

Valid freeze-dried agglutination test batch: Leish DAT Antigen (Royal Tropical Institute, Amsterdam, Netherlands), Lot no 1905 in 5-ml vials was used. Following producer's instructions, the vials were stored at 4ºC-5ºC or at air-conditioned laboratory temperature (23ºC-26ºC) until needed.

Expired freeze-dried agglutination test batches: Leish DAT Antigen (Royal Tropical Institute, Amsterdam, Netherlands), Lot no 1602 (expired four years earlier) was employed.

The original reference FD-DAT was reconstituted according to the manufacturer's instructions with 5 ml of normal saline per vial. Both valid and expired FD-DAT batches were reconstituted with an anti-clumping solution (0.056 M sodium citrate and 0.15 M sodium chloride) supplemented with 1.2% w/v formaldehyde (CSF) instead of normal saline as previously reported to attain a least however eligible promastigote concentration of $1.4x10^7$/ml, instead of the 8.7- $9.0x10^7$/ml originally reported [12]. The valid or expired FD-DAT was stored at 4°C until required.

Execution of the original reference FD-DAT was done as instructed using fetal calf serum (1% v/v) in normal saline. As diluent for both, the improved valid or expired FD-DAT, fetal calf serum was replaced by gelatin at 0.2% wt/vol. in normal saline. The gelatin/saline mixture was heated up to 80°C [7] after which the mixture was left to cool at room temperature. To compare the efficiency to eliminate non-specific agglutination reactions using the original reference or both the improved valid and expired, β-ME (0.8% vol./vol.) or urea (0.3% wt/vol) were used as reducing agents in the FCS or gelatin/saline diluent as described in details earlier [7]. Based on recent observations, supplementation of sodium dodecyl

sulphate (SDS) (0.045 mM) in a gelatin diluent containing NaCl (0.15 M), CaCl2 (0.02 M), KCl (0.05 M) and NaHCO3(0.05M) proved to be highly efficient for the elimination of non-specific agglutination and therefore included in this study as a third reducing agent [13]. Execution of the test with the three FD-DAT antigen types (original reference, improved valid, or improved expired) was carried out using V-shaped well microtiter plates employing initially single dilution testing at 1:25 or 1:100 depending on the objective of the experiment. The test results were read after 18-hour incubation at laboratory temperature (23°C or ≥ 40°C with or without air-conditioning respectively) taking 1: 400 as the cut-off titre for CVL.

LQ-DAT:

L. donovani strain isolated from a VL patient residing in Gedarif area, Eastern Sudan was used as a source for the antigen processing, following the procedures described previously on several occasions [14]. The prepared antigen was then preserved at a concentration of $1.6X10^7$ promastigotes/ml in citrate-saline solution supplemented with 1.2% (wt./vol.) formaldehyde (CSF) to evade auto-agglutination and simultaneously preserve promastigote morphology.

LQ-DAT was also executed according to the improved protocol mentioned above and as previously described in detail [14]. As for the FD-DAT, a titre of 1:400 was also taken as a cut-off for CVL.

rKLO8 ELISA

rKLO8 ELISA was performed as described previously using protein concentrations of 5 ng/well in 0.1 M $NaCO_3$ buffer, pH 9.6 [15,16]. The coated plates were washed with PBS-Tween 20 and then blocked with 3% bovine serum albumin (BSA) in PBS, pH 7.5, for 1 h at room temperature to block nonspecific binding. After washing steps with PBS-Tween 20, 50 ul of diluted sera at 1:800 was added to each well. After another washing steps, the plates were incubated with Peroxidase-conjugated AffiniPure rabbit anti=Dog IgG (H+L) diluted

1:10000 (Immunoresearch Laboratories, USA). Color development performed with the addition of hydrogen peroxide and tetramethylbenzidine (R&D Systems, USA). After 10 minutes of incubation in the dark, 50 µl of 2 N sulfuric acid was added to each well to stop the reaction. The optical densities (OD) were measured at 450 nm using a microplate reader (FLUOstar Omega, BMG LABTECH) with ≥ 0.12 considered indicative for CVL.

Indirect immunolfluorescence (IFAT):

L. infantum promastigote (MON-1) previously isolated from a Croatian dog were harvested at the log phase of growth and washed three times in phosphate buffer saline (PBS). They were then re-suspended in the same buffer at a concentration of 10^7 promastigotes/ml. Ten microliters of the so prepared promastigote suspension was dispended into multispot slides. The slides were let to dry, fixed with methanol, and then washed for 10 min with PBS. They were either stored at +4°C until used, or directly exposed to sera diluted at two-fold concentrations starting with 1: 10 in PBS in a moist chamber at 37°C for 30 min. Excess diluted sera were then removed, and slides were washed thrice in PBS and dried. After drying, promastigotes were let to react with the conjugate (fluoresceinated) rabbit antidog IgG serum (Serotec) for 30 min at 37°C. Afterward washed three times in PBS and dried. Samples showing cytoplasmic or membranous fluorescence with promastigotes at dilutions ≥ 1:80 were considered indicative for CVL [2].

Canine sera: In total 86 sera from male and male populations of dogs were included. Twenty-two were from Sudanese police dogs offered by Captain Hassan, Forensic Affairs of the Ministry of Interior, Khartoum North [14]. This dog group was maintained while not in duty, continuously indoors at the Police Department. Other sixty-four serum samples were from other group of dogs collected during an epidemiological survey in the well-known CVL endemic area of Dalmatia in Croatia [2]. The serum samples from both the Sudanese and

Croatian dog groups were kept at -20°C.

Statistical analysis:

SPSS computer software version 22 was used to measure the variation between performances of the different tests by the One-way ANOVA Test and homogeneity by the Test of Homogeneity of Variances.

Results:

At promastigote concentration of $1.4x10^7$/ ml, the powder antigen reconstituted with citrate-saline/formaldehyde (CSF) as an anti-clumping agent in both the improved valid and expired FD-DAT showed more sharper edged blue spots in the negative control wells implying therefore absence of auto-agglutination in comparison with the reference original (reconstituted at $9.0X10^7$ /ml) with normal saline. On the other hand, unlike the negatives, the CVL positive samples presented a diffuse (mat) layer on the surface of the V-shaped wells indicating agglutination reactions with the original, the improved valid or expired FD-DAT. In comparison with β-ME, both of the alternative reducing agents, urea and SDS, performed excellently using the original reference or either of the two improved variants. Generally, all three reducing agents showed a comparable noticeable reduction in the non-specific reactions (1:200-1:400 down to 1:25-1:100) against the CVL sero-negative sera, but contrarily an increase in the specific versus the CVL sero-positives (1:800-1:6400 up to 1:1600-1:204800) (Table 1). In comparison with urea or SDS, β-ME use proved inconvenient due to the offensive odor and need to follow measures to minimize inhalation.

By taking precautions and further using β-ME in the experiments that followed, clearly negative titres ($\leq$1:100) were recorded for all the 51 sera collected from the Sudanese police dogs (22) and the endemic Croatians (29) against the original reference, the improved valid or expired FD-DAT variant (Table 2). Similar performance for the three FD-DAT variants was also observed in sera of the 15 endemic Croatian dogs who scored clearly positive titres of 1:6400 or higher. At 1:400 titre cutoff performances of the improved valid or expired FD-DAT was highly concordant with each other as well as with the original freeze-dried or liquid in all 19 endemic Croatian dogs; titres $\leq$ 1:200 were recorded with the three test variants in 13 of them. With the exception of one comparable positive titres ranging were measured with the three FD-DAT variants in five of the remaining 6 (Table 3).

Comparable performances were also found between the two improved FD-DAT versions and IFAT as CVL test of choice in Croatia. Except of one out of 13 dogs that tested positive at a low titre of 1:80 all other 12 scored distinctive negative titres ($\leq$ 1:40) in IFAT or of 1:100 against either of the two improved FD-DAT versions. Matching high or medium positive IFAT titres were recorded also with the improved FD-DAT versions in six of the sero-positive endemics.

Based on OD, cutoff value of 0.12, 10 out of the 19 Croatian dogs that tested clearly negative in all four DAT versions showed also negative outcomes with rKLO8 ELISA; two others had very low positive OD values (0.15 or 0.16). All six that showed positive titres in all four DAT versions and in IFAT had also matching rKLO8 ELISA outcomes. Remarkably, in none of the three dogs that tested positive in all four DAT versions as well as in IFAT and KLO8 ELISA, typical CVL symptoms were observed.

The One-way ANOVA Test showed no significant variation between performances of the four DAT versions on the one hand or between the four DAT versions and IFAT or ELISA on the other ($P = 0.142$). The Test of Homogeneity of Variances revealed also significant homogeneity between performances of the six serological tests ($P = 0.009$)

Discussion

Due to a lack of absolute specificity, anti-leishmanial administration is sometimes withheld in symptomatic sero-positive dogs [4]. Based on highly desirable results obtained through intensive evaluation carried out both in endemic and epidemic situations in Bangladesh and Sudan, presence of symptoms typifying VL together with positive LQ-DAT outcomes had adequately justified administration of first-line anti-leishmanials with excellent results [17,18]. Reliable DAT results highly indicative of VL were also reported in Dutch and German dog populations that had overwintered with their owners in the Mediterranean regions with both LQ-DAT and FD-DAT [8,10]. We think due to the unavailability of the test or the very high importation cost involved ($32 per 5-ml vial) no serious attempts were undertaken to assess the merits of applying the DAT as routine CVL diagnosis, particularly in South America and North Africa. Our objective here is to present a number of essential improvements introduced to the FD-DAT which are expected to motivate its application as in the case of VL in the major endemic areas of CVL.

Through successfull replacement of normal saline by formaldehyde/citrate saline (CSF) as an anti-clumping agent for antigen reconstitution, and drastically but eligibly lowering promastigote concentration per unit antigen suspension medium, significant improvement in test feasibility was achieved [12]. Larger volume (12 ml) of an anti-clumping agent CFS could therefore be used, in comparison with the 5-ml normal saline in the original reference FD-DAT. A significant lowering in test cost was therefore achieved from $32.0 down to $12.8 per 5-ml vial. Further sizeable test cost reduction was achieved by revitalizing test batches to their full validity, that were expired four years earlier. So revitalized test batches can be used as valid ones, and with that contributing to further improvement in the test feasibility. Completely different than the original reference FD-DAT, which was using normal saline as re-constituent where a very short shelf-life time of 48-hour was determined,

282 the CSF re-constituted antigens remained valid for at least one year at 4°C for both the
283 improved valid and expired.
284 The use of a single sample dilution at 1:100 for initial screening to help identify potential
285 CVL cases has allowed also economical utilizing of the improved valid and expired FD-DAT.
286 Further testing to full-out titration (≥ 1:6400) for determining the sample end-point titre could
287 then be carried out starting at the 1:400 cut-off titre for CVL.
288 Possibly because of presence of non-specific natural antibodies at levels exceeding those in
289 human host, use of reducing agents in DAT execution seemed therefore indispensable in
290 canine sera. Unlike LQ-DAT and FD-DAT initial versions, β-ME was used as the sole
291 reducing agent [8]. However, because of the associated health hazards and inconvenience in
292 use, reducing agents with minimal or no toxicity such as urea was introduced with success. In
293 this study we also have considered the possibility of introducing a third reducing agent
294 namely the SDS emerging from the highly encouraging results recently observed with human
295 plasma from patients diagnosed with hematological malignancies [13]. As shown in Table 1,
296 all three reducing agents performed satisfactorily in lowering of non-specific agglutination
297 reactions in the CVL negatives while showing increase or maintaining levels in those of the
298 specific noticeably enhancing therefore specificity of all four DAT versions. Both urea and
299 SDS are by far less toxic, convenient and economical to work with than β-ME. The desirable
300 favorable effect of Urea or SDS as compared with β-ME was further clearly reflected on the
301 agreeable outcomes using the two improved FD-DAT versions (Table 2). All 51 dog sera that
302 clearly tested negative with the original reference had scored equally low titres (≤ 1:100) and
303 all 15 that showed highly positive titres with the original did equally so (≥1:6400) with both
304 the improved valid and expired FD-DAT versions.
305 The highly promising performance of these two versions was evidently supported by their
306 concordant outcomes with those of IFAT as well as with an ELISA using a recombinant

antigen (rKLO8) in 19 of the endemic Croatian dogs. Although 5 out of those 19 scored clear positive readings in all of the six sero-diagnostic tests used, no typical CVL symptoms were manifested in any of them (Table 3). This observation was in agreement with other reports implying that suspicion of CVL cannot solely be based on the appearance of the symptoms. Considering warnings reported that treatment failures usually occur should treatment be started after symptoms appear and the common difficulty in demonstrating the parasite, we believe that as in the current strategy for VL management, administration of anti-leishmanials should also be seriously considered for the symptomatic sero-positives CVL cases [18,19]. Since both IFAT and ELISA require certain skills and equipment, LQ-DAT or here improved FD-DAT, because of their lower application cost and simplicity in execution, provide excellent practical diagnostic substitutes.

Based on intensive experience gained during the past three decades at both laboratory and field levels in VL in Sudan and elsewhere, we strongly believe that through the use of the LQ-DAT or FD-DAT version here adequately optimized, and by following a flexible treatment strategy of symptomatic sero-positive dogs, a significant reduction in CVL prevalence can be achieved [17-20].

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Author Contributions

Conceptualization*: el Harith A, Abass E, Martinkovic E, Mansour D, Osman HA*; Data curation: *el Harith A, Osman HA*; Formal analysis: *Osman HA*; Funding acquisition: *el Harith A*; Investigation: *el Harith A, Abass E, Martinkovic E, Mansour D, Osman HA*; Methodology: *el Harith A*; Project administration: *el Harith A*; Resources: *el Harith A, Abass E, Martinkovic*

E, Mansour D, Osman HA; Software: *Osman HA*; Supervision: *el Harith A*; Validation: *el Harith A, Abass E, Martinkovic E, Mansour D, Osman HA*; Visualization: *el Harith A, Abass E, Martinkovic E, Mansour D, Osman HA*; Writing - original draft: *el Harith A*; Writing - review & editing: *el Harith A, Abass E, Martinkovic E, Mansour D, Osman HA*.

Conflict of Interest

The authors declare no conflicts of interest.

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420 **TABLES:**

421 **Table 1: Comparative efficiencies of β-mercaptoethanol (β-ME), urea, and sodium**
422 **dodecyl sulphate (SDS) as reducing agents for elimination of non-specific agglutination**
423 **reactions in canine sera**

FD-DAT	Number of dogs	Serum pretreated with the reducing agent: Untreated	β-ME	Urea	SDS
Improved valid	2	1:200, 1:400	1:50, 1:100	1:25, 1:50	1:25, 1:50
	2	1:800	1:3200	1:3200, 1:6400	1:3200
	2	1: 12800, 1:51200	1: 25600, 1:204800	1: 25600, 204800	1: 25600, 1:204800
Improved expired	2	1:200, 1:400	1:50	1:25, 1:50	1:25, 1:50
	2	1:800	1:3200	1:3200	1:3200
	2	1:12800, 1:51200	1:25600, 1:204800	1:25600, 1:204800	1:51200, 1:204800
Original reference	2	1:200, 1:400	1:50, 1:100	1:50	1:25, 1:50
	2	1: 800	1:1600, 1:3200	1: 3200, 1:6400	1: 3200
	2	1:12800, 1:51200	1:25600, 1:204800	1:25600, 1:204800	1:51200, 1:204800

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Table 2: Validity of the improved valid or expired freeze-dried agglutination test in comparison with the original reference for detection of leishmaniasis in the canine reservoir

Number of Dogs	Freeze-dried direct agglutination test (FD-DAT) reading * (titre)		
	Improved valid	Improved expired	Original reference
51	≤ 1: 100	≤1: 100	≤ 1: 100
1	1: 400	1: 400	1: 800
1	1: 800	1: 400	1: 1600
1	1: 400	1: 400	1: 1600
1	1: 800	1: 800	1:1600
1	1: 1600	1: 1600	≥1: 6400
1	1: 1600	1: 1600	≥1:3200
1	1:1600	1:1600	1: 6400
15	≥1: 6400	≥1: 6400	≥1: 6400

* β-ME was used as the reducing agent.

433 **Table 3: Performance of the improved valid and expired freeze-dried agglutination test**

434 **in comparison with the original reference (FD-DAT), the liquid DAT version (LQ-DAT),**

435 **indirect immunofluorescence (IFAT), enzyme-linked immune-sorbent assay (ELISA)**

436 **and manifestation of leishmaniasis (CVL) symptoms in 19 Croatian dogs**

Number of dogs	FD-DAT (titre)			LQ-DAT (titre)	IFAT (titre)	KLO8 ELISA (OD)	CVL symptoms
	Improved valid	Improved expired	Original reference				
9	≤1:100	≤1:100	≤1:100	≤1:100	1:40	0.03 – 0,09	Asymptomatic
1	≤1:100	≤1:100	1:200	1:200	1:80	0.03	Asymptomatic
1	≤1:100	≤1:100	≤1:100	≤1:100	1:40	0,15	Dermatitis
1	≤1:100	≤1:100	≤1:100	≤1:100	1:40	0.16	Asymptomatic
1	≤1:100	≤1:100	≤1:100	≤1:100	1:40	0.54	Conjunctivitis
1	1:3200	1:6400	1:6400	1:6400	1:320	1.2	Asymptomatic
1	1:51200	1:51200	1:25600	1:25600	1:5120	1.93	No data
1	1:51200	1:102400	1:204800	1:204800	1:10240	1.89	Asymptomatic
1	1:25600	1: 51200	1: 51200	1:51200	1:640	1.68	No data
1	1:204800	1:204800	1:102400	1:102400	1:2560	0.4	No data
1	1:3200	1:6400	1:6400	1:3200	1:80	1.59	Asymptomatic

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