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Selection of a Unified Standard Complement Fixation Method for Nation-Wide Application to Restore Inter-Laboratory Harmony to the Diagnosis of Ruminant Brucellosis

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Abstract: Despite being technically demanding, the complement fixation test (CFT) continues to be massively applied as a global quantitative confirmatory test for the diagnosis of brucellosis in reference laboratories. Variations in CFT protocols applied in different laboratories worldwide under diverse conditions have led to some inter-lab inconsistency of results. The aim of this work was to restore inter-lab harmony at the national level by selection of a CFT method from five standard CFT variants, *viz*. the American, the British, the French/ European Union, the German and the modified Australian techniques. Known positive and negative sera from domestic ruminants (198 cows, 66 buffalo cows, 95 ewes, 39 female goats and 98 she-camels) as well as control dilutions from the national Egyptian standard anti-*Brucella abortus* serum (equivalent to the OIE International Standard Serum) were used for evaluation. The quantitative data of CFT were used in a two-way analysis of variance (ANOVA) followed by a post hoc test, where significant differences between methods and between animal species were detected. The lowest detection limits for CFT assays were determined. Using C-ELISA in lieu of a gold standard, the performance characteristics of the CFT methods were estimated. These included agreement, relative sensitivity and specificity, false positive and negative rates, likelihood ratios of positive and negative results, diagnostic odds ratio and the area under the receiver operating characteristics (ROC) curves representing accuracy. The CFT results were compared and fully discussed. Both the CFT EU and CFT US performed very well in comparison to the other CFT assays in terms of sensitivity specificity balance. For technical reasons, the CFT US was selected as a unified method for nation-wide application to avoid inter-lab disagreement. Practical means for bringing order to harmonize CFT results were discussed.

Key words: Brucella · CFT · Sensitivity · Specificity · LR · DOR · Accuracy · ROC

INTRODUCTION

Despite its technical sophistication, the complement fixation test (CFT) lingers as a worldwide quantitative confirmatory test for brucellosis in reference laboratories. The test detects mainly IgG_1, characteristic of infection and some IgM, but not IgG_2 or IgA [1]. This bestows CFT a particular diagnostic niche in small ruminants [2]. Where CFT has a standardized system of unitage and the test correlates well with culture results and active immune response against *Brucella* spp. [3], it is still prescribed by the World Organization for Animal Health [4,5] for international trade.

Variations in CFT protocols applied in different laboratories all over the world have led the OIE to recommend certain technical condition ranges for carrying out the reagent titration and the test proper [5] aiming for international reproducibility of results. These recommendations include, for instance, the range of complement units producing 50% or 100% hemolysis (1.25 to 2 $C'H_{100}$, or 5 to 6 $C'H_{50}$) and hemolysin units (2 to 5 H_{100}) to be used in the test. The antigen recommended for the test should have about 2% packed cell volume to give 50% fixation with 1/200 of the OIE International Standard (anti-Brucella) Serum (OIEISS), showing 100% fixation at the lower serum dilutions. Not only that, but the OIE Terrestrial Manual [4, 5] specified the conditions of serum heat inactivation prior to the test proper in terms of dilution with veronal buffer, temperature and time of inactivation.

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The enactment of CFT used to go through ups and downs due to the lack of standardization until the mid-nineties [6], when a remarkable progress started thanks to the adoption of good quality measures and the resulting harmonization of results. In the year 2000, the OIE Reference Laboratory for Brucellosis in the United Kingdom (AHVLA, formerly VLA) launched a series of pan-European proficiency ring trials for bovine brucellosis serology using different panels of sera to boost intra- and inter-lab diagnostic accuracy among national reference laboratories of the European Union (EU) member states [7]. The Community Reference Laboratory for Brucellosis (currently the EU Reference Laboratory for Brucellosis) of ANSES (French Agency for Food, Environmental and Occupational Health Safety), assisted by AHVLA [8], prepared an EU SOP [9] intended for harmonization of results and international unit (IU) conversion.

The majority of national labs rely massively on the semi-quantitative rivanol test rather than the quantitative complement fixation test for confirmation of serologically positive animals. Unlike CFT which is recommended by the OIE [5] as an international confirmatory test, the rivanol test is not even mentioned, where its reliability in small ruminants is questioned [10]. The Egyptian control program for brucellosis is based on vaccination of young seronegative animals as well as test and slaughter of seropositive ones with compensation paid to the owners by the General Organization for Veterinary Services. To avoid too much and too low condemnation of animals especially in the middle of a control policy and an economic crisis, a unified standard for serologic confirmation should be adopted to synchronize lab results on the national level. Egyptian scientists use different CFT methods/ protocols of varying sensitivity [11-15], on top of carrying out the test on a limited scale that favors changeable results. To make matters worse, CFT reagents are labile and require accurate titration especially complement [16].

The aim of the present work was to select, on technical grounds, a standard CFT method that is sensitive enough to detect the vast majority of infected animals especially ruminants infected with *Brucella melitensis*. Five main standard CFT variants were tried, *viz*. the American, the British, the French/ EU, the German and the modified Australian techniques.

MATERIALS AND METHODS

Animals: A total of 496 serum samples from different ruminant species were selected from the serum collection of the Department of Brucellosis Research, Animal Health Research Institute, Dokki, Giza, Egypt (Table 1). Most of these sera were serologically positive and belonged to animals in the Nile Delta governorates. Most of the bovine, ovine and caprine positive sera had a history of *Brucella melitensis* biovar 3 infection [15]. There was no history of vaccination against brucellosis. Only some animals were reported to have late abortion and retained placenta.

Serological Tests

Buffered Acidified Plate Antigen (BAPA) and Brucellosis Card (BCT) Tests: Antigens for the BAPA and BCT 8% were obtained from the Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Egypt. The BCT antigen (3% cells) for small ruminants was prepared, standardized and verified in the Department of Brucellosis Research, Animal Health Research Institute, Dokki, Giza, Egypt according to the American method [16,17]. The BAPA was performed according to the OIE Terrestrial Manual [4,5]. The BCT was implemented in large ruminants (8% cells) as described by Alton *et al.* [16] and in small ruminants (3% cells) according to Mikolon *et al.* [17] as currently adopted by the NVSL, USDA [18]. Although BAPA and BCT are qualitative tests, their results were recorded as scores of 1+ to 4+ according to the degree of agglutination for the sake of comparison with other quantitative tests.

Complement Fixation Test (CFT) Versions: Antigen for the American CFT was imported from NVSL/DBL, USDA, USA, while that of the British CFT was imported from AHVLA, New Haw, Addlestone, Surrey KT15 3NB, UK. Working dilutions of antigen for the other three CFT methods were made from the American antigen according to titration results against the OIEISS.

Complement and hemolysin were prepared and preserved according to Alton *et al.* [16] and coping with Hennager (2004) (H. E. Stowell, personal communication, November 22, 2010) [19]. These were titrated according to the CFT method in question as mentioned in the next paragraph in addition to Gillard [20] and Brown [21] for the British method (P. Lowe, personal communication, November 23, 2010). Sheep RBCs were collected on Alsever's solution from an adult healthy ram serologically negative to brucellosis. These were standardized to 2, 2.5 and 3% suspensions in VBS in line with the CFT method in question referring to Gillard [22] for the British method (L. Perrett, personal communication, November 22, 2010).

Table 1: Epidemiologic data of ruminant sera included in CFT comparison and selection

Animal spp.	Breed	Age*	Population	Governorate	No.
Cows	Hybrid/ Friesian	1-4	Small/ large herds	Beheira/ Sharkia/ Monofia/ Gharbia	198
Buffalo cows	Native	1.5-5	Individuals/ small herds	Beheira/ Sharkia/ Monofia/ Gharbia	66
Ewes	Native	1-4	Individuals/ small flocks	Kafr El-Sheikh/ Beheira/ Sharkia/ Monofia/ Gharbia	95
Female goats	Native	1-3	Individuals/ small flocks	Beheira/ Sharkia/ Giza	39
She-camels	Fellahi	2-3	Individuals	Sharkia	98
Total animals					496

* Age in years

Table 2: Interpretation and conversion of CFT titers of different methods to international complement fixing units per milliliter of the OIE International Standard Serum (OIEISS)

Interpretation	ICFTU/ ml	CFT US titers	CFT GDR titers	CFT UK/FR/EU titers	CFT AU titers
Negative	10	1/2.5	4/5	2/2	4/2
Positive	20	1/5	4/10	2/4	4/4
	40	1/10	4/20	2/8	4/8
	80	1/20	4/40	2/16	4/16
	160	1/40	4/80	2/32	4/32
	320	1/80	4/160	2/64	4/64
	640	1/160	4/320	2/128	4/128
	1280	1/320	4/640	2/256	4/256
	2560	1/640	4/1280	2/512	4/512

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

Table 3: Conversion factors for CFT titers of different methods

Degree of fixation	CFT US titers	CFT GDR titers	CFT UK/FR/EU titers	CFT AU titers
25% (+)	4	1.25	4.125 to 4.16	3.125
50% (++)	5	1.5	5	3.75
75% (+++)	6	1.75	5.83	4.375
100% (++++)	7	2	6.65	5

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

American 4/143 or 2/200 = 1000 ICFTU/ml

British, French, EU 4/150 or 2/200 = 1000 ICFTU/ml

Australian 4/200 or 2/308 = 1000 ICFTU/ml

Six (actually five) standard CFT methods were compared. These included, in no particular order, the American CFT by Hennager (current version) (H. E. Stowell, personal communication, November 15, 2010) [23], the British CFT by Brinley Morgan *et al.* [24] and Gillard (current version) [25] (L. Perrett, personal communication, August 10, 2010), the French CFT [26], which is the EU CFT [9] (F. Melzer, personal communication, April 29, 2013), the German CFT [27] and Anonymous [28] (A. Habashi, personal communication, April 8, 2013) and the modified Australian CFT [16]. Regardless of the method in question, warm fixation of complement at 37°C was adopted as cold fixation was unacceptably slow. For the German and the European techniques normally built on cold fixation, complement fixation for the test proper was carried out for 60 minutes at 37°C and for 30 minutes at 37°C respectively. Results of CFT versions were converted to ICFTU/ml and interpreted as in Tables 2 and 3. The positive cutoff point for all CFT techniques including the modified Australian was ≥ 20 ICFTU/ml [5].

Competitive ELISA: Commercial multispecies competitive ELISA kit (SVANOVIR® Brucella-Ab C-ELISA), produced by Svanova Biotech AB, Uppsala, Sweden. This kit uses *Brucella abortus* smooth lipopolysaccharide antigen, horseradish peroxidase conjugated antibovine IgG monoclonal antibodies and tetramethylbenzidine in substrate buffer containing H_2O_2.

The kit was validated according to the kit instructions, the validation guidelines of the ISO/IEC 17025 [29], Crowther [30] and OIE Terrestrial Manual [31]. The test was performed according to the kit instructions. The percent inhibition (PI) was calculated from the formula:

PI = 100 – [(Mean OD samples×100)/ (Mean OD Conjugate control)]

The status of a test sample was determined as follows:

PI Status ≥ 30% was considered positive. If < 30%, the test was negative.

Statistical Analyses: All the following analyses were performed using IBM® SPSS® Statistics, Version 21, IBM Corporation, 2012, under the environment of Windows® 8.1, Microsoft Corporation.

Two-Way Analysis of Variance (ANOVA): The titers of CFT methods were converted to IU/ml of serum. The OD readings of C-ELISA were expressed as PI. These raw data were statistically tested for the normal distribution using SPSS different parameters. Unfortunately, the assumption of normal distribution of data was rejected as indicated by the SPSS' Shapiro-Wilk test at p value < 0.05. Data were then transformed using log base 10 + 1 to follow normal distribution. A two-way ANOVA with post hoc test using the least significant difference (LSD) were used to study the statistical significant differences in the means of CFT methods as an independent factor and their effect on the results (the dependent factor).

Kappa (κ) Agreement and Relative Sensitivity/ Specificity: The kappa (κ) agreement of CFT methods with C-ELISA in lieu of the gold standard was used to assess the matching of results at $p < 0.05$. Relative sensitivity/ specificity pairs were also calculated.

Performance Indicators of Serologic Tests: This included the calculation of FPR (false positive rate), FNR (false negative rate), LR+ (likelihood ratio of a positive test), LR- (likelihood ratio of a negative result) and DOR (diagnostic odds ratio). These were calculated according to McGee [32], Loong [33] and Macaskill *et al*. [34].

Receiver Operating Characteristics (ROC) Curves: Considering the C-ELISA as the gold standard, ROC curves expressing the sensitivity versus the false positive rate were plotted for all CFT methods. Data were obtained from ROC curves including the area under the curve (AUC) representing accuracy, the best positive cutoff points and the equivalent true positive/ false positive rates according to Hanley and McNeil [35].

RESULTS AND DISCUSSION

Technical Variation of CFT Methods: Effect on Diagnostic Performance: The diagnostic performance of a test is a reflection of its sensitivity and specificity combined. The technical parameters affecting the diagnostic performance of six standard CFT variants are revealed in Tables 4 and 5 as factors related to reagent titration and factors regarding the proper test respectively. Titration processes of CFT biological components (Table 4) are mainly affected by the total volume of reagents (macro-/ micro-method) and their final dilutions, the target hemolysis percentage (50 or 100%) and the way it is detected either visually or spectrophotometrically and the number of units (1.25 to 6) and concentration (half/ full unit) of reagents.

Hemolysis, as a means for visualization of complement fixation to the hemolytic system, is mainly dependent on the ratio of the heat-labile complement, the fragile RBCs and the incubation temperature and duration. This necessitates titration of complement on every day's test given the fact that the fragility of an erythrocyte batch increases day by day. The larger the total volume in titration, the smaller the pipetting error. The packed cell volume of erythrocytes and the target hemolysis percent determine the complement concentration required as half or full unit, as well as the visibility of results. The target hemolysis of 50% read spectrophotometrically and the working dilution of complement calculated via log-log graphing ensure higher accuracy compared to target hemolysis of 100% read visually and the working dilution of complement calculated without a graph paper.

The test proper (Table 5) is primarily influenced by the serum heat inactivation conditions (prior dilution in CFT diluent if any, temperature and time), the final dilution/ concentration of reagents including the sheep RBCs and the incubation time (30 or 60 minutes). Doubling inactivation time eliminates anticomplementary activity but reduces sensitivity [36] by selection for fixation of complement via different immunoglobulin isotypes [5]. IgM denatures at 65°C for 15 minutes [37]. Temperatures just below 65°C tend to inhibit IgM. This is the case in CFT US and in CFT GDR and CFT AU for small ruminants. Prior dilution of serum for heat treatment also tends to minimize the frequency of anticomplementary activity. Another key factor of CFT sensitivity is the final concentration of antigen (expressed as the packed cell volume of the working dilution). The final concentrations of complement and RBCs in the test are generally a consequence of titration

Table 4: Technical conditions affecting reagent titration of different CFT versions for diagnosis of livestock brucellosis

Criteria		OIE guidelines	CFT US	CFT UK	CFT FR	CFT EU	CFT GDR	CFT AU
Reference		OIE Terrestrial Manual [5]	Hennager [23]	Brown [21] & Gillard [20, 22)	Norme Française [26]	Anonymous [9]	Anonymous [28]	Alton *et al.* [16]
Complement titration	Reagents' total volume (ml)	macro-method preferred	4.8	0.125 (micro-method)	1	1	3	4
	C' initial dilutions	-	1/100 to 1/800	1/10 to 1/280	1/150 to 1/1000	1/125 to 1/500	1/40 to 1/200	1/656.25 to 1/1750
	C' dilution volumes (ml)	-	1.2	0.025	0.4	0.2	0.5	1.5
	C' final dilution factor	-	1.2/4.8 = 1/ 4	0.025/0.125 = 1/ 5	0.4/1 = 1/ 2.5	0.2/1 = 1/ 5	0.5/3 = 1/ 6	1.5/4.0 = 1/2.67
	C' final dilution times	-	400 to 3200	50 to 1400	375 to 2500	625 to 2500	240 to 1200	1750 to 4666.7
	With/ without antigen	either way	with	both compared	with	with	with	without
	Fixation time & temperature	-	60 min. at 37°C	30 min. at 37°C	30 min. at 37°C	30 min. at 37°C	60 min. at 37°C	30 min. at 37°C
	Reading of hemolysis % by matching with standard	-	spectrophoto-metrically	visually	visually	visually	visually	spectrophoto-metrically
	Target hemolysis %	H_{50} preferred	H_{50}	H_{100}	H_{50}	H_{50}	H_{100}	H_{50}
	Valid range of hemolysis %	-	10-90	76-100	10-90	10-90	100	10-90
	Working dilution calculation	-	log-log graph	no graph	no graph	no graph	no graph	semi-log graph
	Target C' units (minimum hemolytic dose)	1.25 to 2 $C'H_{100}$, or 5 to 6 $C'H_{50}$	5 $C'H_{50}$	1.25 to 2 $C'H_{100}$	6 $C'H_{50}$	6 $C'H_{50}$	2 $C'H_{100}$	5 $C'H_{50}$
Hemolysin titration	Reagents' total volume (ml)	-	4	0.125	0.125	0.125	3	4
	Hemolysin initial dilutions	-	1/1500 to 1/32000	1/500 to 1/6000	1/250 to 1/6000	1/250 to 1/6000	1/500 to 1/12000	1/500 to 1/10000
	Hem. dilution volumes (ml)	-	0.8	0.025	0.025	0.025	0.5	1.0
	Hem. final dilution factor	-	0.8/4.0 = 1/ 5	0.025/0.125 = 1/ 5	0.025/0.125 = 1/ 5	0.025/0.125=1/ 5	0.5/3 = 1/ 6	1/ 4
	Reading of hemolysis %	-	Visually	Visually	Visually	visually	visually	Visually
	Target hemolysis %	100%hemolysis	H_{100}	H_{100}	H_{100}	H_{100}	H_{100}	H_{100}
	Valid range of hemolysis %	-	30-80	76-100	76-100	76-100	30-80	30-80
	Working dilution calculation	-	via graph	no graph	no graph	no graph	no graph	via graph
	Target hemolysin units	2-5 H_{100}	1.25 H_{100}	5 H_{100}	2 H_{100}	2 H_{100}	4 H_{100}	1.25 H_{100}
Antigen titration	Reagents' total volume (µl)	-	150	100	125	125	150	100
	Antigen initial dilutions	-	1/00, 1/200 to 1/700	1/100, 1/200 to 1/600	1/100, 1/200 to 1/600	1/100, 1/200 to 1/600	1/ 2, 1/ 4 to 1/128	1/100, 1/200 to 1/600
	The antigen of about 2% PCV should give 50% fixation with 1/200 of the OIEISS, showing 100% fixation at the lower serum dilutions.		The antigen is standardized to give 2/200 of the OIEISS by each method					The antigen is adjusted to 1/320 of the OIEISS.
	Antigen dilution volumes (µl)	-	25	25	25	25	25	25
	Antigen final dilution	-	25/150 = 1/ 6	25/100 = 1/ 4	25/125 = 1/ 5	25/125 = 1/ 5	25/150 = 1/ 6	25/100 = 1/ 4
	Target antigen units	-	1 H_{50}	2 H_{100}	1 H_{50}	1 H_{50}	1 H_{50}	2 H_{100}

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT, C' = complement, H_{50} = 50% hemolysis, H_{100} = 100% hemolysis, PCV = Packed cell volume, OIEISS = OIE International Standard Serum against *Brucella abortus*

Table 5: Technical conditions affecting the test proper of different CFT versions for diagnosis of livestock brucellosis

Criteria		OIE recommendations	CFT US	CFT UK	CFT FR	CFT GDR	CFT EU	CFT AU
Reference		OIE Terrestrial Manual [5]	Hennager [23]	Gillard [25]	Norme Française [26]	Anonymous [28]	Anonymous [9]	Alton *et al.* [16]
Heat inactivation conditions	Serum dilution in VBS	either undiluted at	1/ 5	1/ 2	undiluted	undiluted	undiluted	undiluted
	Temperature (bovines)	60°C±2°C for 30 minutes,	60-63°C	58°C	59±1°C	56°C	59±1°C	58°C
	(sheep & goats)	or diluted 1/2 at 58°C±2°C	(optimum 62°C)	60°C		(Africans 60°C)		62°C
		for 50 minutes				62-63°C		
	Time in minutes		30±5	50 (bovines), 30 (small ruminants)	30	30	30	30
Total volume (µl) of test proper		Technique bound (100-125)	150	100	125	150	125	100
Serum (heat inactivated)	Volume (µl)	25	25	25	25	25	25	25
	Initial dilution	1/ 2	1/ 5	1/ 4	1/ 4 or 1/ 2	1/ 5	1/ 4 or 1/ 2	1/ 4
Antigen	Volume (µl)	25	25	25	25	25	25	25
	Working dilution of native Ag		-	1/500	1/10	1/10	1/10	1/10 1/100
	PCV of working dilution	-	0.088%	0.04%	0.15%	0.275%	0.15%	0.088%
	Final dilution in the test	25/125 = 1/ 5	25/150 = 1/ 6	25/125 = 1/ 5	25/125 = 1/ 5	25/150 = 1/ 6	25/125 = 1/ 5	25/100 = 1/ 4
Complement	Volume (µl)	25	50	25	25	50	25	25
	Final dilution	25/125 = 1/ 5	50/150 = 1/ 3	25/125 = 1/ 5	25/125 = 1/ 5	50/150 = 1/ 3	25/125 = 1/ 5	25/100 = 1/ 4
	Fixation time & temperature	either 30 m' at 37°C, or 14-18 hr at 4°C	60 m' at 37°C	30 m' at 37°C	16-20 hr at 5±3°C *	60 m' at 37°C *	16-20 hr at 5±3°C *	30 m' at 37°C
Sheep RBCs (in hemolytic system)	Percentage	2, 2.5 or 3%	2%	3%	2.5%	2%	2.5%	3%
	Volume (µl)	25-50	50	25	50	50	50	25
	Final dilution	1/5 - 1/10	25/150 = 1/6	12.5/125=1/10	25/125 = 1/5	25/150 = 1/6	25/125 = 1/5	12.5/100 = 1/8
	Final percentage	0.4, 0.5 or 0.6%	0.33%	0.3%	0.5%	0.33%	0.5%	0.375%
	Incubation time (m')	30	30	30	30	30	30	30
Positive cutoff		≥ 20 ICFTU/ml	≥ $1/5^{+}$	≥ $1/4^{++}$	≥ $1/4^{++}$	≥ $1/10^{++++}$	≥ $1/4^{++}$	≥ $1/4^{++++}$

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

* For CFT GDR and CFT EU originally built on cold fixation, complement fixation for the test proper was carried out for 60 minutes and 30 minutes at 37°C respectively.

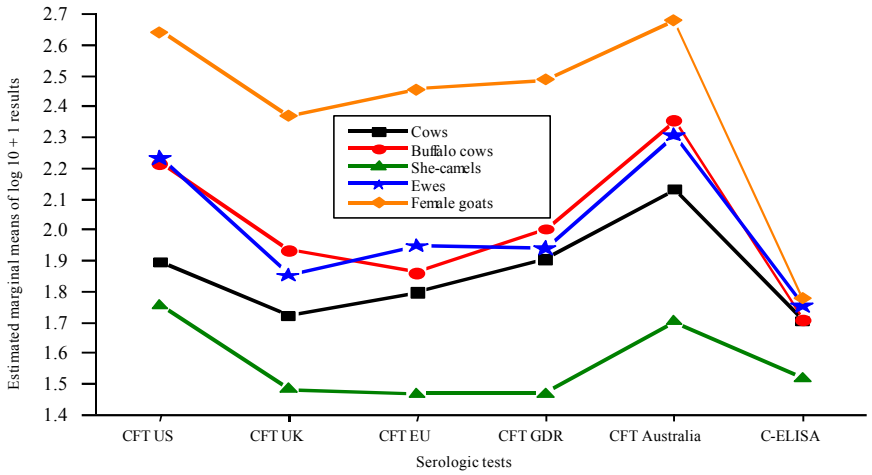

Fig. 1: Animal inter-species variation expressed as the mean differences among them in serologic results

Table 6: Analysis of variance for factors affecting the results of serologic tests

Factor	F score	Significance (p value)
Animal species	43.921	0.000
Serologic test method	16.888	0.000
Animal species vs. test method	1.514	0.067

*: significant at $p < 0.05$ with confidence interval of 95%

Table 7: Post hoc test using the least significant difference (LSD) to reveal variation in serologic results among different animal species

Animal species (I)	Animal species (J)	Mean difference (I-J)	Standard error	Significance (p value)
Cows	She-camels	0.326*	0.047	0.000000
Buffalo cows	Cows	0.153*	0.054	0.004618
	She-camels	0.479*	0.060	0.000000
Ewes	Cows	0.146*	0.047	0.002110
	She-camels	0.472*	0.054	0.000000
Female goats	Cows	0.542*	0.066	0.000000
	Buffalo cows	0.389*	0.076	0.000000
	Ewes	0.396*	0.072	0.000000
	She-camels	0.868*	0.072	0.000000

*: significant at $p < 0.05$ with confidence interval of 95%.

Insignificant variations were excluded.

and the pivotal antigen concentration. The ratios of these biologicals together with that of hemolysin are very much interdependent. Hemolysin has relatively little effect, if any, because it is used in an ample amount to sufficiently sensitize RBCs for the action of complement.

Comparison of Standard CFT Methods for the Diagnosis of Ruminant Brucellosis: For the sake of fair comparison and to minimize technical difference of CFT versions examined, only warm fixation was followed despite the fact that cold overnight fixation is more technically sound. Apart from being time consuming, cold fixation is notorious for anticomplementary activity [16], a disadvantage that reduces the number of samples testable by CFT. On the other hand, the main disadvantage of the relatively rapid warm fixation is the potential occurrence of prozones, a problem that can be easily overcome by making enough serial dilutions of sample [16].

For preliminary comparison of the standard CFT techniques, a statistical approach was followed. The quantitative data of CFT were used in a two-way analysis of variance (ANOVA) to detect the effect of CFT assays and animal species on the results. The F scores (Table 6) indicated highly significant statistical variation at $p < 0.05$ and confidence interval of 95% resulting from the difference in animal species as well as the test method each as an independent factor, the interaction between each other and their effect on the test results.

Table 8: Post hoc test using LSD to reveal variation among different CFT versions and competitive ELISA

Test category (I)	Test category (J)	Mean difference (I-J)	Standard error	Significance (p value)
CFT US	CFT UK	0.2527*	0.05855	0.000016
	CFT EU	0.2137*	0.05909	0.000304
	CFT GDR	0.1868*	0.05882	0.001506
	C-ELISA	0.3498*	0.05921	0.000000
CFT EU	C-ELISA	0.1362*	0.05948	0.022142
CFT GDR	C-ELISA	0.1630*	0.05921	0.005948
CFT AU	CFT US	0.1199*	0.05882	0.041554
	CFT UK	0.3726*	0.05855	0.000000
	CFT EU	0.3336*	0.05909	0.000000
	CFT GDR	0.3067*	0.05882	0.000000
	C-ELISA	0.4697*	0.05921	0.000000

CFT US = American CFT, CFT UK = British CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT, *: significant at $p < 0.05$ with confidence interval of 95%.

Insignificant variations were excluded.

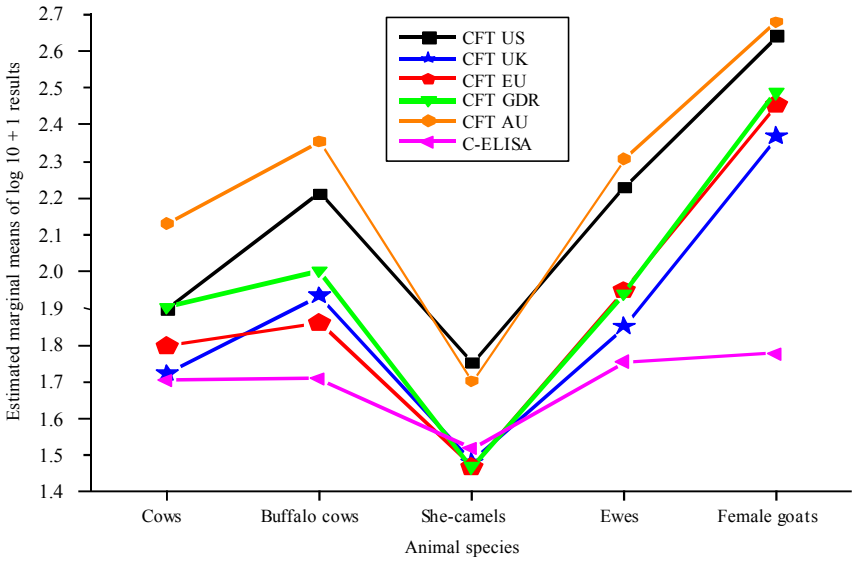

Fig. 2: Variation among CFT versions expressed as the mean differences among them in serologic results

To investigate the source of variation within animal species, a post hoc test using the least significant difference (LSD) was resorted to (Table 7 and Figure 1). Goats and she-camels differed extremely from the other animal species at $p < 0.05$ and confidence interval of 95%. Cows varied slightly from buffalo cows and ewes, but greatly from goats and she-camels. There was no statistical variation (0.904427) between buffalo cows and ewes. The difference of she-camel results could be attributed to the unique nature of camel serum IgG including 25% complement fixing IgG_1, while the remainder IgG_2 and IgG_3, devoid of the light chains and the CH(1) domain, are non-complement fixing [38]. The variation of goat results might be resorted to the nature of serum IgG being composed of 55% complement fixing IgG_1 and 45% non-complement fixing IgG_2 [39]. Regarding cattle and sheep, IgG is similar in both species including 50% complement fixing IgG_1, IgG_2 of varying concentrations and IgG_3 [40]. Buffalo IgG has two identical subclasses of IgG_1 and IgG_2 [41].

To trace variation among CFT methods, a post hoc test using the LSD was carried out (Table 8). Excluding C-ELISA where the nature of its result outcome (PI) is different from those of CFT versions (ICFTU/ml), the American and modified Australian CFT techniques were significantly different from other CFT methods (Figure 2) at $p < 0.05$ and confidence interval of 95%. Results of the European CFT techniques including the CFT UK, CFT FR, CFT EU and CFT GDR expectedly showed no significant variation. To reveal detailed statistical variation of every CFT version in different animal species, a post hoc test was performed using the LSD (Table 9 and Figure 1).

Table 9: Post hoc test using LSD to reveal variation within animal species versus different CFT versions and competitive ELISA

Test	Animal spp. (I)	Animal spp. (J)	Mean difference (I-J)	Standard error	Significance (p value)
CFT US	Buffalo cows	She-camels	0.462*	0.147	0.002
		Cows	0.316*	0.132	0.016
	Ewes	Cows	0.334*	0.116	0.004
		She-camels	0.480*	0.133	0.000
	Female goats	Cows	0.745*	0.162	0.000
		Buffalo cows	0.429*	0.187	0.022
		She-camels	0.890*	0.175	0.000
		Ewes	0.411*	0.176	0.020
CFT UK	Cows	She-camels	0.241*	0.114	0.035
	Buffalo cows	She-camels	0.453*	0.147	0.002
	Ewes	She-camels	0.370*	0.130	0.004
	Female goats	Cows	0.646*	0.162	0.000
		Buffalo cows	0.434*	0.187	0.020
		She-camels	0.888*	0.175	0.000
		Ewes	0.517*	0.174	0.003
CFT EU	Cows	She-camels	0.329*	0.114	0.004
	Buffalo cows	She-camels	0.393*	0.147	0.008
	Ewes	She-camels	0.479*	0.137	0.000
	Female goats	Cows	0.658*	0.162	0.000
		Buffalo cows	0.594*	0.187	0.001
		She-camels	0.987*	0.175	0.000
		Ewes	0.508*	0.179	0.004
CFT GDR	Cows	She-camels	0.629*	0.114	0.000
	Buffalo cows	She-camels	0.728*	0.147	0.000
	Ewes	She-camels	0.663*	0.133	0.000
	Female goats	Cows	0.582*	0.162	0.000
		Buffalo cows	0.483*	0.187	0.010
		She-camels	1.210*	0.175	0.000
		Ewes	0.548*	0.176	0.002
CFT AU	Cows	She-camels	0.430*	0.114	0.000
	Buffalo cows	She-camels	0.651*	0.147	0.000
	Ewes	She-camels	0.606*	0.133	0.000
	Female goats	Cows	0.547*	0.162	0.001
		She-camels	0.977*	0.175	0.000
		Ewes	0.371*	0.176	0.035

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

*: significant at $p < 0.05$ with confidence interval of 95%.

Insignificant variations were excluded.

Serologic variation of CFT methods among animal species in ascending order was respectively revealed as follows by CFT UK, CFT EU, CFT US, CFT GDR and CFT AU. The American CFT was relatively moderate in showing variation among animal species.

Technical comparison of the standard CFT methods was based on the determination of their performance indicators, *viz*. analytical sensitivity, agreement with C-ELISA as a sensitive highly specific test [5], sensitivity and specificity relative to C-ELISA, FPR and FNR, likelihood ratios and diagnostic odds ratio (Tables 10 to 12 and Figure 3). Such diagnostic metrics accurately quantify minor differences among assays necessary for successful selection of the most appropriate.

Increasing concentrations of 5 to 1000 ICFTU/ml of the anti-*Brucella* national standard serum homologous to the OIEISS were tested with CFT versions to determine the minimum analytical sensitivity of each method (Table 10). For every CFT method, one pre-test serum dilution was made to assess detection limits as low as might be. At pre-test dilutions, the highest analytical sensitivity was achieved by CFT US with detection limit as low as 10 ICFTU/ml, followed by CFT AU (18 ICFTU/ml), CFT GDR (22 ICFTU/ml) and both CFT UK and CFT EU (25 ICFTU/ml). The minimum analytical sensitivities as revealed by the first dilution of each method were 20 (CFT US), 25 (CFT AU), 35 (CFT GDR) and 40 (CFT UK and CFT EU) ICFTU/ml, indicating

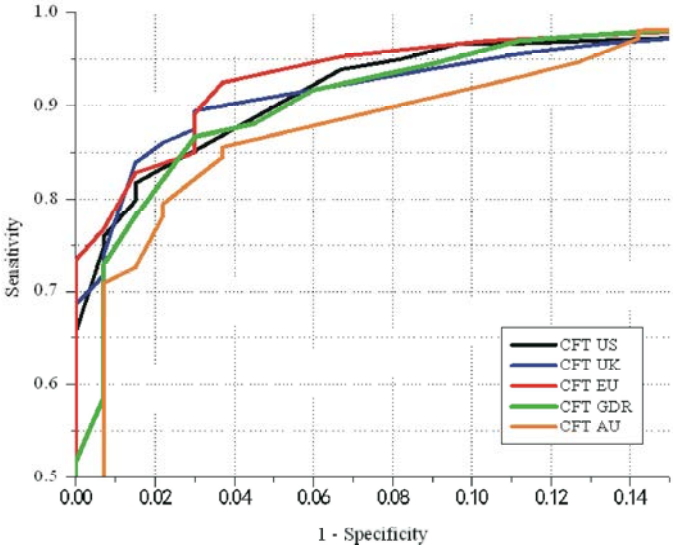

Fig. 3: ROC curves showing diagnostic performance of CFT versions in ruminants

Table 10: Minimum analytical sensitivities of serological tests including different warm microtechniques of CFT using known dilutions of the national Egyptian standard anti-*Brucella abortus* serum equivalent to the OIEISS

Anti-*Brucella* national standard serum dilutions expressed as ICFTU/ml	BAPA	BCT	CFT US	CFT UK	CFT EU	CFT GDR*	CFT AU
5	0	0	0	0	0	0	0
7.5	0	0	0	0	0	0	0
10	0	0	1/2.5	0	0	0	0
12.5	0	0	2/2.5	0	0	0	0
15	0	0	3/2.5	0	0	0	0
18	+	0	2.5	0	0	0	2/2
20	+	+	1/5	0	0	0	3/2
22	+	+	1/5	0	0	1/2.5	4/2
25	+	+	2/5	1/2	1/2	2/2.5	1/4
30	+	+	3/5	3/2	2/2	4/2.5	2/4
35	+	+	4/5	4/2	4/2	3/5	4/4
40	+	+	1/10	4/4	1/4	4/5	1/8
50	+	+	2/10	4/8	2/8	4/10	1/16
100	+	+	2/20	2/32	1/16	2/40	2/32
200	+	+	2/40	1/64	2/16	4/40	2/64
500	+	+	3/80	1/128	1/32	2/80	2/128
1000	+	+	3/160	1/256	4/64	3/160	3/256
Minimum analytical sensitivity (ICFTU/ml) at a pre-test dilution	18	20	10	25	25	22	18
Minimum analytical sensitivity (ICFTU/ ml) at the first test dilution			20	40	40	35	25

BAPA = buffered acidified plate antigen test, BCT = brucellosis card test, CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

* Unlike the original German CFT that adopts cold fixation of complement, the German technique in this study was performed using warm fixation as well as the other CFT techniques under investigation.

** CFT titer of 100% fixation at a serum dilution of 1/2.5

*** Analytical sensitivity is the ability of an assay to detect traces of the analyte (antibody activity expressed as international units).

maximum analytical sensitivity of CFT US. The relative low detection limit of CFT US could be attributed to the use of a diluted antigen of 0.088% packed cells (Table 5). Both CFT US and CFT AU used the exact concentration of antigen and yet the former had a lower detection limit. This could be explained by the final dilution of the antigen in the test method, where it was diluted 6 times in the former compared to 4 times in the latter (Table 5).

The ability of CFT methods to correctly identify sera from 7 ewes and 4 female goats bacteriologically proven infected with *Brucella melitensis* was tested (Table 11). All CFT versions identified all infected animals except for the British and the German techniques which missed two and one animal respectively. This again confirms the sensitivity of CFT US, CFT AU and CFT EU in descending order in small ruminants.

Table 11: Serologic titers of different CFT warm microtechniques in sheep and goats bacteriologically proven to be infected with *Brucella melitensis*

Serial No.	Animal species/ sex	BAPA	BCT	CFT US	CFT UK	CFT FR/EU	CFT GDR	CFT AU
1.	Ewe	3+	4+	80^{+}	2	4^{++}	2.5	8^{+++}
2.	Ewe	2+	2+	160	32	256^{+}	40	128^{+++}
3.	Ewe	3+	4+	160	16	64	20	64^{+++}
4.	Ewe	3+	4+	320^{++}	128^{+}	256	160	256
5.	Ewe	3+	2+	320	32	128	40	128^{++}
6.	Ewe	2+	2+	40^{++}	64	256^{++}	80	128
7.	Ewe	2+	2+	640	64	256^{+++}	160^{+}	256
8.	Female goat	1+	1+	10^{++}	8^{+}	32^{+}	5	32^{+}
9.	Female goat	3+	4+	640	256	512	320	256
10.	Female goat	4+	4+	640	4^{+}	8	5^{++}	16^{++}
11.	Female goat	2+	2+	40^{+}	64^{+}	256^{++}	80	128

BAPA = buffered acidified plate antigen test, BCT = brucellosis card test, CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

Table 12: Agreement with C-ELISA of CFT versions and their diagnostic performance characteristics

Results of CFT versions		C-ELISA results* -	+	Agreement (κ value **)	Relative Se (%)	Relative Sp (%)	FPR (%)	FNR (%)	LR+	LR-	DOR	AUC
CFT US	-	100	2	0.801 ± 0.031	98.0	91.3	8.7	2	11.264	0.022	527.9	0.983
	+	34	359									
CFT UK	-	110	6	0.840 ± 0.028	94.8	93.7	6.3	5.2	15.047	0.055	271.3	0.978
	+	24	355									
CFT EU	-	112	7	0.846 ± 0.028	94.1	94.1	5.9	5.9	15.949	0.063	257.5	0.981
	+	22	354									
CFT GDR	-	110	5	0.845 ± 0.028	95.7	93.7	6.3	4.3	15.190	0.046	326.5	0.979
	+	24	356									
CFT AU	-	85	2	0.707 ± 0.037	97.7	88.0	12	2.3	8.142	0.026	311.4	0.973
	+	49	359									

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

-: number of negative cases, +: number of positive cases, *: gold standard

**: agreement with C-ELISA at $p < 0.05$ with confidence interval of 95% ± standard error

Se = sensitivity, Sp = specificity, FPR = false positive rate, FNR = false negative rate, LR+ = likelihood ratio of a positive test (the probability of an animal who has the disease testing positive divided by the probability of an animal who does not have the disease testing positive), LR- = likelihood ratio of a negative result (the probability of an animal who has the disease testing negative divided by the probability of an animal who does not have the disease testing negative), DOR = diagnostic odds ratio (summarizes the diagnostic accuracy of the test as a single number that describes how many times higher the odds are of obtaining a test positive result in a diseased rather than a non-diseased animal), AUC = area under the ROC curve representing accuracy at confidence interval of 95%.

Taking the C-ELISA as the reference standard, kappa (κ) agreement was calculated (Table 12) for matching of the index CFT assays with the comparator. Landis and Koch [42] characterized κ values < 0 as indicating no agreement and 0- 0.20 as slight, 0.21- 0.40 as fair, 0.41- 0.60 as moderate, 0.61- 0.80 as substantial and 0.81- 1 as almost perfect agreement. All CFT assays except CFT AU agreed almost perfectly with C-ELISA. The maximum relative sensitivity was expressed by CFT US (98%) and the maximum relative specificity was shown by CFT EU (94.1%). The minimum summation of FPR and FNR was revealed by CFT GDR (10.6%) and CFT US (10.7%).

Likelihood ratios quantify the change in the certainty of the diagnosis conferred by test results. LRs > 1 argue for the disease diagnosis and the bigger the number, the more convincingly the disease is suggested, while LRs between 0 and 1 argue against the disease and the closer the number to 0, the less likely the disease [32]. High LR+ ratio reflects the assay's good ability to predict the true disease status with corresponding low LR- ratio (Table 12). The best LR+ ratio was achieved by CFT EU (15.949) followed by CFT GDR (15.190), CFT UK (15.047), CFT US (11.264) and finally CFT AU (8.142), while the best LR- ratio was attained by CFT US (0.022) followed by CFT AU (0.026), CFT GDR (0.046), CFT UK (0.055) and CFT EU (0.063).

The diagnostic odds ratio summarizes the diagnostic accuracy of the index CFT assay as a single number that describes how many times higher the odds are of obtaining a test positive result in a diseased rather than a

Table 13: Comparison of diagnostic performance metrics of different CFT assays arranged in descending order of superiority

	κ	Se	Sp	FPR	FNR	LR+	LR-	DOR	AUC
CFT US	4th	1st	3rd	3rd	1st	4th	1st	1st	1st
CFT UK	3rd	4th	2nd	2nd	4th	3rd	4th	4th	4th
CFT EU	1st	5th	1st	1st	5th	1st	5th	5th	2nd
CFT GDR	2nd	3rd	2nd	2nd	3rd	2nd	3rd	2nd	3rd
CFT AU	5th	2nd	4th	4th	2nd	5th	2nd	3rd	5th

κ = agreement, Se = sensitivity, Sp = specificity, FPR = false positive rate, FNR = false negative rate, LR+ = likelihood ratio of a positive test, LR- = likelihood ratio of a negative result, DOR = diagnostic odds ratio, AUC = accuracy

CFT US = American CFT, CFT UK = British CFT, CFT FR = French CFT, CFT GDR = German CFT, CFT EU = European CFT, CFT AU = modified Australian CFT

non-diseased animal [34]. The higher the DOR, the better the test performance (Table 12). The best DOR values was that of the CFT US (527.9), followed by the CFT GDR (326.5), CFT AU (311.4), CFT UK (271.3) and CFT EU (257.5).

ROC curves were plotted for CFT assays (Figure 3) to evaluate the overall assay performance and to determine accuracy by estimation of the area under each of the ROC curves [43]. The AUC measures how well the test separates the positive from negatives without reference to a particular decision threshold [35]. AUCs of 0.9-1, 0.8-0.9, 0.7-0.8 and 0.6-0.7 indicate excellent, good, fair and poor test respectively, while an AUC of 0.5-0.6 designates an invalid test [35]. The accuracy of all CFT assays was excellent being above 0.95. The highest accuracy was achieved by CFT US (0.983).

To separate the highly overlapping ROC curves resulting from similar assay performance (Figure 3), it was necessary to stretch the horizontal X-axis scale 5 times the Y axis. The closer the ROC curve to the vertical axis, the better the test performance [43]. At first glance to the tracks of ROC curves, it is noticed that the curves of CFT EU, CFT UK and CFT US represent the best assay performances starting at around the range of 70% sensitivity. Close observation to the convexity of each of these three curves leads to the finding that the curve for CFT US is the most concave without dents away from the Y axis resulting from occasional drops in specificity at some diagnostic thresholds. This makes CFT US preferable in terms of ROC performance all the way up as sensitivity increases.

Selection of a CFT Method and Reasons Behind the Choice: Reviewing Table 13, both CFT US and CFT EU performed very well in comparison to the other CFT assays in terms of sensitivity specificity balance. CFT US and CFT EU were ranked as No. 1 in 5 and 4 performance indicators respectively. CFT EU was ranked 5th in 4 performance parameters. CFT US achieved the highest accuracy expressed as DOR and AUC. Considering the cons and pros, comparison of the diagnostic performance of different standard CFT methods has led the authors to prefer the American (USDA) version for the following technical reasons. The method ensures strict conditions for very accurate titration of complement, the most delicate reagent whose concentration greatly affects the test sensitivity and reproducibility. This is fulfilled by performing the complement titration in large macro-volumes of 4.8 ml (Table 4) to minimize pipetting errors. The target end point of complement titration is a mid-point of 50% (not 100%) hemolysis ensured by spectrophotometric rather than naked-eye reading. The 50% point is easier to fine-tune as it is free to go up and down (49 or 51%) compared to the 100% point of hemolysis that has no way but to go down. The accurate dose of complement causing exact 50% hemolysis is determined using log-log graph papers, one graph for every complement dilution. The graph validation conditions also offer quality assurance for the titration results; where in case that the graph is invalid, complement titration is repeated with different dilutions and a new lot of sheep RBCs. The precise concentration of sheep RBCs for either reagent titration or test proper is adjusted spectrophotometrically by the hemoglobin content, not by the packed cell volume. Likewise, titration of hemolysin is also done in macro-volumes of 4 ml (table 5) and the working dilution is estimated through a graph paper. It is noteworthy that most, but not all, of the abovementioned technical features are also found in the modified Australian technique of Alton *et al.* [16].

As to the test proper of the USDA method, the heat inactivation conditions ensure minimal anticomplementary activity of test sera by prior dilution of sera in CFT diluent at 1/5 (or 1/2.5) and inactivation at the maximum permissible temperature range of 60-63°C (Table 6) above which destruction of the heat labile IgM occurs [37]. The test proper uses 50 µl of the working dilution of complement (personal communication, 2010), which is twice the volume suggested by the OIE [4] with fixation time of 60 minutes, which is double the OIE recommended

time. This, followed by the addition of 50 µl of hemolytic system, allows for comparatively more efficient fixation of complement and hence, higher diagnostic specificity needed for the main quantitative confirmatory test at the national level. It is noteworthy that enhanced specificity, as revealed by the ROC curve, suits the current epidemiological situation of brucellosis in Egypt, where disease control rather than eradication is being practiced.

The analytical sensitivity of the American method was determined as 10 ICFTU/ml starting at a test dilution of 1/2.5 using the national serum based on the OIEISS. The conversion factors for converting titers with different degrees of fixation (25, 50, 75 and 100%) to ICFTU/ml are integral numbers 4, 5, 6 and 7 respectively (Tables 3 and 4) rendering calculation easier.

Bringing Harmony to Intra and Inter-Lab Results of CFT at the National Level: The World Organization for Animal Health [5] recommended that any country using the CFT on a national level should obtain agreement among the different laboratories performing the test to use the same method in order to obtain the same level of sensitivity. In keeping with this recommendation, the central national diagnostic veterinary lab (AHRI) performing CFT on routine basis, suggests the adoption of the standard American method at the national level for the reasons discussed earlier. In addition, AHRI produces and standardizes the secondary national standard equivalent of the OIEISS for distribution to other governmental labs on demand.

CONCLUSION

Among the five standard CFT techniques compared under conditions of this investigation, the American (USDA) version was selected to be the national standard method for confirmation of brucellosis in ruminants. Global harmonization of CFT results by the use of OIEISS as a means for standardization together with the follow up of OIE recommendations may not be adequate. The additional adoption of a single universal CFT method for carrying out the test, with different cutoffs if necessary, may be required to overcome its technical complications and method-to-method variation.

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