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Molecular and Serological Diagnosis of Buffalo's Brucellosis in Salahaldeen Governorate

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ABSTRACT

The aims of the study were to determined incidence ratio of buffalos brucellosis in Salahaldeen governorate and evaluating the efficiency of serological tests used in current study. For this purpose 135 buffalos (adult males, pregnant females and aborted females) were tested.
From each animal two blood samples were collected, one for PCR test and other for serological tests (Rose Bengal test, tube agglutination test, mercaptoethanol test and ELISA test).
The incidence ratio of buffalo brucellosis in Salahaldeen governorate were 14.8% in PCR test, while in serological tests were 21.4%, 13.3%, 16.2% and 7.4% for rose Bengal test, tube agglutination test, mercaptoethanol test and ELISA test respectively. Highest positive and negative agreement recorded between rose Bengal test and mercaptoethanol test were 34.9% & 63.7% .
The current study recorded highest sensitivity recorded in rose Bengal test (75%), highest Specificity in ELISA (97.1%). Highest postive predictive values s and negative predictive values s recorded by tube agglutination test were: 77.7% and 96.2%

التشخيص الجزيئي والمصلي لداء البروسيلا عند الجاموس في محافظة صلاح الدين

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كلية الطب البيطري/ جامعة تكريت

الخلاصة

هدفت الدراسة تحديد انتشاء الاصابة بداء البروسيلات في الجاموس في محافظة صلاح الدين وتحديد كفاءة الاختبارات المصلية المستخدمة في التشخيص، ولهذا الغرض اخضع للدراسة 135 (اناث مجهضة ، حوامل، اناث وذكور بالغة). من كل حيوان اخذ نموذجين من الدم : الاول لاجراء اختبار تفاعل البوليميرات المتسلسل والثاني فصل المصل منه واجري عليه اربع الاختبارات المصلية (اختبار وردية البنكال، اختبار التلازن الانبوبي، اختبار المركابتوايثانول واختبار الاليزا).
بلغت النتائج الايجابية الكلية اعتمادا على اختبار تفاعل البوليميراز المتسلسل 14.8% اما الاختبارات المصلية فقد اعطت نتائج ايجابية بلغت 21.4% و 13.3% و 16.2% و7.4% لاختبار وردية البنكال, واختبار التلازن الانبوبي واختبار المركابتوايثانول واختبار الاليزا على التوالي. سجلت اعلى نسبة توافق بين اختبار وردية البنكال واختبار المركابتوايثانول اذ بلغت ، 34.9% و 63.7% للتوافق الايجابي والسلبي على التوالي. اعطى اختبار وردية البنكال اعلى حساسية بلغت 75% واعطى اختبار الاليزا اعلى نوعيةً بلغت 97.1% وقد كان اختبار التلازن في الانابيب الاعلى على مستوى التنبؤ الايجابي والسلبي اذا بلغا 77.7% و 96.2% على التوالي.

الكلمات المفتاحية:
البروسيلا، الجاموس، الاختبارات المصلية، تفاعل البوليمرات المتسلسل.
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Introduction:

The Brucellosis term refers to infection in both humans and animals caused by many species of the genus Brucella, mainly *Brucella abortus, B. melitensis and B. suis.* (OIE,2016). This disease cause highly economic loss due to abortion of pregnant animals, decrease of fertility rate and highly cost of treatment and Immunization programs (CFSPH,2007).
The disease transmission among animals and to human by direct or indirect contact with contamination sources which include: abortive embryos, vaginal fluids, embryonic membranes, urine and milk (Radostits *et al.*,2007).

Brucella infects mainly mature animals (cows, sheep, goats, pigs, dogs, horses, buffaloes, reindeer, weeds and camels). The disease is usually asymptomatic in young animals and non-pregnant females, whereas in pregnant adult females develops aplacentitis usually resulting in abortion last third months of pregnancy (OIE,2016). In Adult males, brucella causes orchitis and/or epididymitis and Hygromas, usually involving leg joints (FAO,2003; Radostits *et al.*,2007).

Brucellosis diagnosed by direct and indirect methods (serological methods), serological tests some time give false negative or false positive reaction due to vaccination or passive immunity, or cross reaction and in carrier animals (Corbel & MacMillan,1996).

Brucella can be isolate from blood, bone marrow, vaginal discharge, embryonic membranes, semen, milk, and synovial fluid of aborted animals. In aborted fetuses brucella isolated from the liver, spleen, lung, and stomach contents. Form infected animal carcasses can be isolated from Lymph nodes, spleen, uterus, testis, joint exudate, and abscesses (Alton,1990) . (PCR) can directly detect DNA of the causative agent ,therefore it used for establishing current infection status. This assay is not only sensitive and fast but also safe.

Amis of study : determined incidence ratio of buffalos brucellosis in Salahaldeen governorate by using of serological tests and genetic method. Then study of serological tests efficiency (Sensitivity, Specificity, positive predictive values and negative predictive values) depend on PCR test

Materials and methods:

- **Animal samples**:

  buffaloes blood samples collected from different areas Salahaldeen province. sex and physiological state of buffalos as shown in table (1).

Table (1): sex and physiological state of buffaloes

Sex and physiological state of buffaloes	Number of animal
Aborted females	21
Pregnancy females	62
Adult females	40
Males	12
Total	135

- **Samples collection**

  From each animal two blood samples were collected, first with EDTA (for DNA extraction) and the second without EDTA (for serological tests)

- **Molecular technique:**

  a- DNA extraction: DNA extraction by use of the kit (QIAamp DNA Mini Kit- QIAGEN)

  b- DNA mixture is used for amplification: as table (2)

Table (2) Compounds used in the preparation of Reaction Mixture

Compounds used in preparation of Reaction Mixture	Volume (microliter)
Taq PCR Master Mix KIT (Qiagen, Germany) Which contain Taq DNA Polymerase (2.5 Unit), PCR Buffer with 3mM MgCL2, 200µMdNTP (Qiagen, Germany).	25
Primer A (B. A. Forword) 5/ ACG, CAG, TCA, GAC, GTT, GCC, TAT,3/ (Funakoski, Japan)	0.3 from 100pM Solution.
Primer B(B.A. Reverse) (BCSP31) 5/ TCC, AGC, GCA, CCA, TCT, TTC, AGC, CTC, 3/(Funakoski, Japan)	0.3 from 100pM Solution.
DNA Template	3
DNA free water (Qiagen, Germany)	21.4
Total	50

c- Timer program of the thermocycler as in table (3).

Table (3) Timer program of the thermocycler

Stage	Temperature (c°)	Time (mints)	Cycles (nombers)
First Denaturation step	94	4 mints	1
Denaturation step	94	1 mint	30
Primer-annealing step	60	1 min	
DNA extension step	72	1 mint	
Final DNA extension step	72	10 mints	1
End Temperature	4	___	___

Serological test:

1- Rose Bengal test (slide agglutination methods) : applied according to (Alton *et al*.,1975) by the use of the kit provided by (Spinreact company- Spain) , appear of agglutination refer to positive result.
2- Tube agglutination test (TAT): applied by the use of the kit provided by (Morganville company- USA) company. With European system in dilution according to (Alton *et al*.,1975).
3- Mercaptoethanol test : applied by the use of the kit provided by (Morganville company- USA) company. With European system in dilution by Mercaptoethanol solution (prepare by 8.5gram NaCl with 7.14 ml stock solution of 2- Mercaptoethanol, then complete the volume to 1L by distal water) according to (Alton *et al*.,1975).
4- ELIZA : applied by the use of the kit provided by svanova (SVANOVIR®Brucella-Ab I-ELISA)

statistical analysis:

- Positive agreement = $\frac{N.of\ sampls\ gave\ positive\ results\ in\ first\ test}{N.of\ sample\ gave\ positive\ results\ in\ second\ test} X100$
- Negative agreement = $\frac{N.of\ sampls\ gavenegative\ results\ in\ first\ test}{N.of\ sample\ gave\ negative\ results\ in\ second\ test} X100$ (Kahya et al.,2010).
- Sensitivity= $\frac{True\ positive}{True\ positive+false\ negative} X100$
- Specificity= $\frac{True\ negative}{True\ negative+false\ positive}$X100
- positive predictive values = $\frac{True\ positive}{True\ positive+false\ positive}$ X100

- negative predictive values = $\frac{True\ negative}{True\ negative+false\ negative}$ X100 (Elwood, 1998).

Results and discussion:

PCR test :

Brucella spp detection from buffaloes in survey study is (14.8%). The isolation ratio differs according to Sex and physiological state of animals which are: in Aborted females (61.9%), Pregnancy females (6.4%), Adult females (5.0%) and in Males (8.3%). Table (4), figure (1)

Table (4): PCR Results

Sex and physiological state of animals	Number of animals	Number positive cases	Positive ratio
Aborted females	21	13	61.9%
Pregnancy females	62	4	6.5%
Adult females	40	2	5.0%
Males	12	1	8.3%
Total	135	20	14.8%

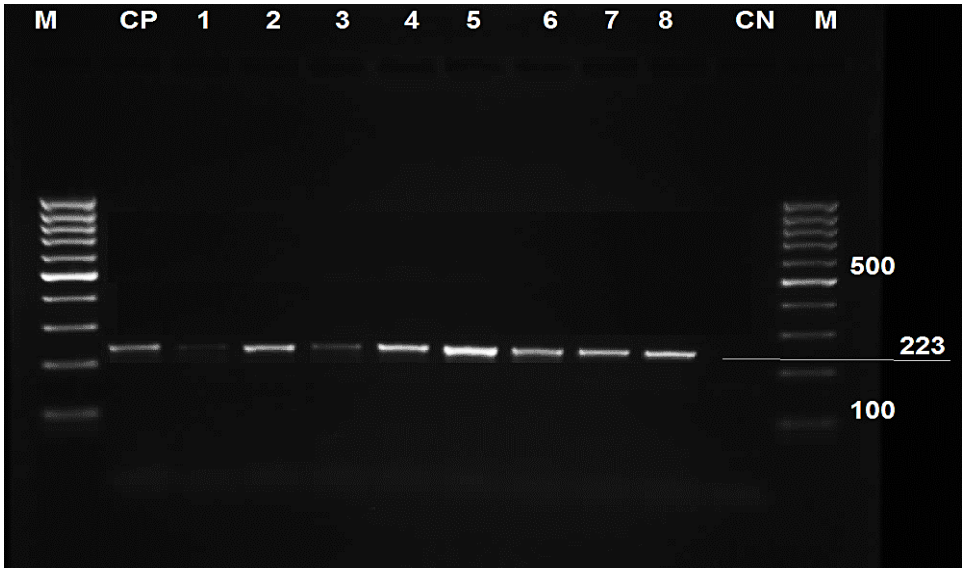

Figure (1): Electrophoresis on 2 % a garose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker, CP control positive, CN: control negative, wells 1-8 positive samples band in size 223 bp.

According to the PCR test, infection ratio of brucellosis in buffaloes was 14.8%. This ratio is more than ratio that recorded by (Kanta *et al*.,2014) which are 5.45% , and the ratio recorded by (Mohmed, 2015) which 7.8%. that differences may be attributed to the variance of geographic location of study, type of sample and physiological state of animals.

In present study, the highest percentage was observed in aborted buffalos which are 61.9%. brucellosis is the main abortion reason in buffaloes (Wareth *et al*.,2015). The males may be infected or appear as carrier animal and play role in spreading the infection (Corbel& MacMillan, 1996).

Madboly *et al* (2014) show positive ratio were 9.3% and all samples that positive to serological test were positive to PCR test.

In our study many cases give positive result in PCR with out clinical signs . that's Probably to be treated or carrier animals or exposed to sub infected dose of brucella.

Serological tests

- **Rose Bengal tests**: The infection ratio of brucellosis in buffalos by using rose Bengal test were (21.4%). Table (5) shows positive results according to sex and physiological state.

Table (5): Rose Bengal tests

Sex and physiological state of animals	Number of animals	Number positive cases	Positive ratio
Aborted females	21	6	28.5%
Pregnancy females	62	9	14.5%
Adult females	40	11	27.5%
Males	12	3	25%
Total	135	29	21.4%

In current study the infection ratio 21.4%(29:135). This ratio is more than ratio that recorded by (Abdul Al- Hussain & Thaer, 2012) which 7.2%. and ratio recorded by (Al-abdaly, 2012) which is 13%. And less that ratio that recorded by (Abd Al-Azeem *et al*.,2012) which 53%. That's may be due to difference geographic location of study, physiological condition of animal. Prevalence of brucella were Increasing with years (Mohmed,2015).

Rose Bengal test can give a false positive result due to antibody come from vaccination or passive immunity or cross reaction with other bacteria like: *Vibrio cholera*، *Escherichia coli*، *Escherichia hermanni*، *Yersinia enterocolitica*، *Salmonella urbana* and *Francisella tularensis* (Corbel, & MacMillan, 1996).

The current study showed that highest positive ratio was recorded in pregnancy buffalo, that agreement with (Abdul Al- Hussain, & Thaer,2012). That's due to high sensitivity of pregnant animal to infected with Brucella, due to high concentration of erythritol which a stimulant for the growth of Brucella (Radostits *et al*.,2007).

Appearance of antibrucella antibody may be refer to previous infection, vaccination and cross reaction, these case is more communally in pregnant animals due to age, stress causes by pregnant and immunization program.

Tube agglutination test (TAT): according to Tube agglutination test the ratio of brucellosis in buffaloes were: in aborted buffalos: 52.3% , in Pregnancy buffalo: 8.0% , in adult females: 5.0% , while the least ratio is recorded in males which are 0.0%.Table(6).

Table (5): Tube agglutination test Results

Sex and physiological state of animals	Number of animals	Number positive cases	Positive ratio
Aborted females	21	11	52.3%
Pregnancy females	62	5	8.0%
Adult females	40	2	5.0%
Males	12	0	0.0%
Total	135	18	13.3%

The result of tube agglutination test showed infection ratio was 13.3%. This result is more than ratio recorded by (Abdul Al- Hussain, & Thaer, 2012). that's maybe due to different of study time, time between infection and sample taken and physiological state of buffalo.

The highest ratio was recorded in aborted females, that's may be refer to acute infection.

Tube agglutination test more efficiency to detection IgM than IgG (Alton, 1990). And its gave false negative result in early stage and chronic stage of infection (Radostits *et al*.,2007).

Mercaptoethanol test results: the present study reveals that IgG titer increases in 22 cases out of 135 with ratio of 16.2%. Table (6) explain the differences results according to Sex and the physiological state of animals.

Table (6): Mercaptoethanol test Results

Sex and physiological state of animal	Number of animals	Number positive cases	Positive ratio
Aborted females	21	3	14.2%
Pregnancy females	62	9	14.5%
Adult females	40	9	22.5%
Males	12	1	8.3%
Total	135	22	16.2%

The presence study reveal that high positive result recorded in adult female in compared with other studied group. That may be refer to presence of IgG which come from previous infection or vaccine or chronic infection. Mercaptoethanol can distraction of IgM and leave IgG (McGiven *et al.*,2003).

ELISA test: the present study shows difference in the ratio of infection with brucellosis (Aborted animals: 9.5%, Pregnancy females: 6.4%, Adult females: 5% and Males: 0%) .Table (7). The Figure (2) Summarizing of positive results of serological tests that used in current study.

Table (7): ELISA Results

Sex and physiological state of animal	Number of animals	Number positive cases	Positive ratio
Aborted females	21	2	9.5%
Pregnancy females	62	4	6.4%
Adult females	40	2	5%
Males	12	0	0%
Total	135	10	7.4%

infection ratio of brucellosis by ELISA test were 7.4% that ratio is less than ratio recorded by (Madboly *et al*.,2014). That's may be due to different of geographic location of study, number of samples, time of sample taken**,** age of animals and physiological state of animals. ELISA kite used in current study apple to detected only immunoglobulin IgG. IgG increase in case of chronic infection, old infection and after vaccination (Alton *et al*.,1975).

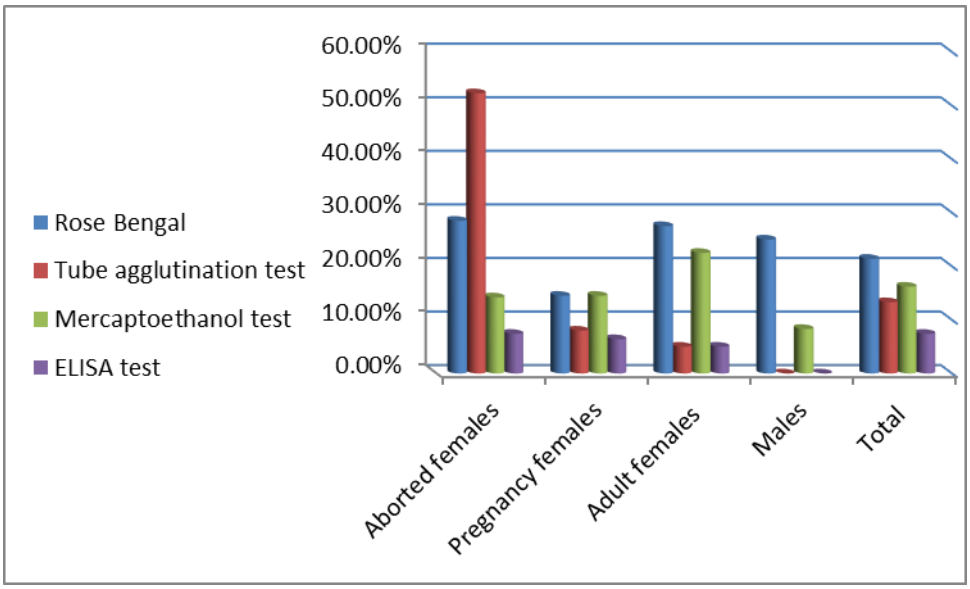

Figure (2) ratio of positive results in serological tests

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- **Agreement between Rose Bengal test with other serological tests**: statistical analysis of the results was showed positive agreement between rose Bengal and Tube agglutention test, Mercaptoethanol test, and ELISA test were: 28.5%, 34.9% and 15.8% respectively while negative agreement between rose Bengal and Tube agglutention test, Mercaptoethanol test, and ELISA test were: 53.8%, 63.7% and 57.6% respectively. Table (8), Figure (3).

Table (8): Agreement between Rose Bengal test with other serological tests

Rose Bengal Test	Tube agglutention test		Mercaptoethanol test		ELISA test	
	+ve cases	-ve cases	+ve cases	-ve cases	+ve cases	-ve cases
+ve=63 -ve=72	18	117	22	113	10	125
Positive agreement	28.5%		34.9%		15.8%	
Negative agreement		53.8%		63.7%		57.6%

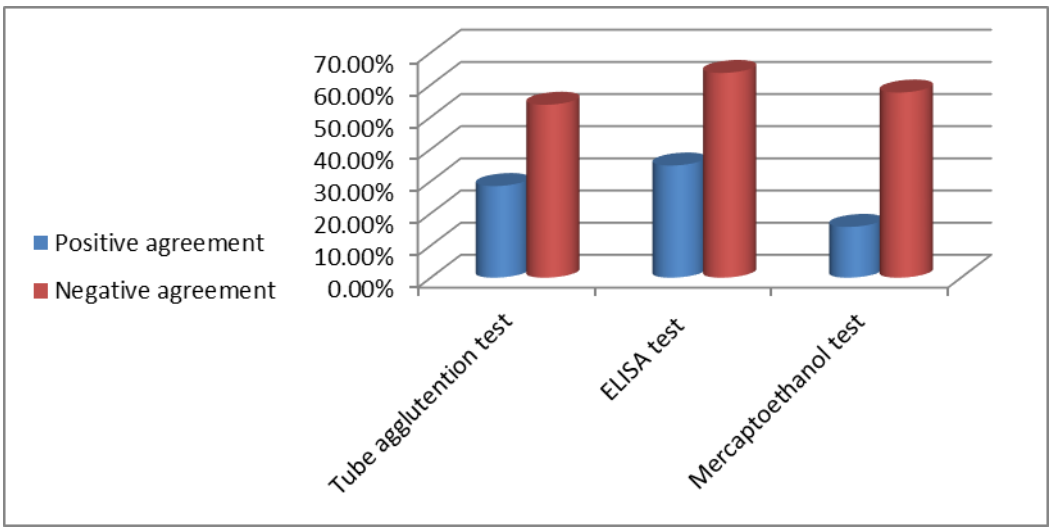

Figure (3): Agreement between Rose Bengal test with other serological tests

- **Agreement between Mercaptoethanol test and ELISA**: positive agreement was: 45.5% while negative agreement 90.4%. table (9)

Table (9): Agreement between Mercaptoethanol test, and ELISA

Mercaptoethanol Test	ELISA test	
	+ve	-ve
+ve=22 -ve= 113	10	125
Positive agreement	45.5%	
Negative agreement	90.4%	

Appearance of agreement between positive result of rose Bengal with positive result of other serological test refer to the ability of rose Bengal to detection both types of Ab (IgG and IgM). Rose Bengal characterized by easy speed and low cost, and this test reveals antibodies of types IgG and IgM (Radostits *et al*.,2007).

Appearance of agreement between positive result of rose Bengal with negative result of other serological test may be reveal to either cross reaction of rose Bengal or less efficiency of other serological test.

Evaluation of serological test depending on PCR test:

sensitivity of serological test were: 75%, 50%, 32.1% and 25% for rose Bengal, tube agglutination test, mercaptoethanol and ELISA respectively. while the Specificity were60.7%, 96.2%, 87.8% and 97.1% for rose Bengal, tube agglutination test, mercaptoethanol and ELISA respectively

positive predictive values of serological tests were: 33.3%, 77.7%, 40.9% and 70% for for rose Bengal, tube agglutination test, mercaptoethanol and ELISA respectively while **negative predictive values of serological tests were**: 90.2%, 96.2%, 83.1% and 83.2% for rose Bengal, tube agglutination test, mercaptoethanol and ELISA respectively. Table (10, 11, 12, 13), Figure (4)

Table (10): evaluation result of Rose Bengal test

Result of rose Bengal test	Result of PCR test				Total
	Positive		Negative		
	True positive	21	False positive	42	63
	False negative	7	True negative	65	72
Total		28		107	135

Sensitivity : 75%, Specificity: 60.7%, positive predictive values : 33.3%, negative predictive values :90.2%

Table (11): Evaluation result of Tube agglutination test

Tube agglutination test	Result of PCR test				Total
	Positive		Negative		
	True positive	14	False positive	4	18
	False negative	14	True negative	103	117

Table (12): Evaluation result Mercabtoethanol test

Mercabtoethanol test	Result of PCR test				Total
	Positive		Negative		
	True positive	9	False positive	13	22
	False negative	19	True negative	94	113
Total		28		107	135

Sensitivity : 32.1% , Specificity: 87.8%, positive predictive values : 40.9%, negative predictive values :83.1%

Table (13): Evaluation result of ELISA

ELISA test	Result of PCR Test				Total
	Positive		Negative		
	True positive	7	False positive	3	10
	False negative	21	True negative	104	125
Total		28		107	135

Sensitivity : 25% , Specificity: 97.1%, positive predictive values : 70%, negative predictive values :83.2% .

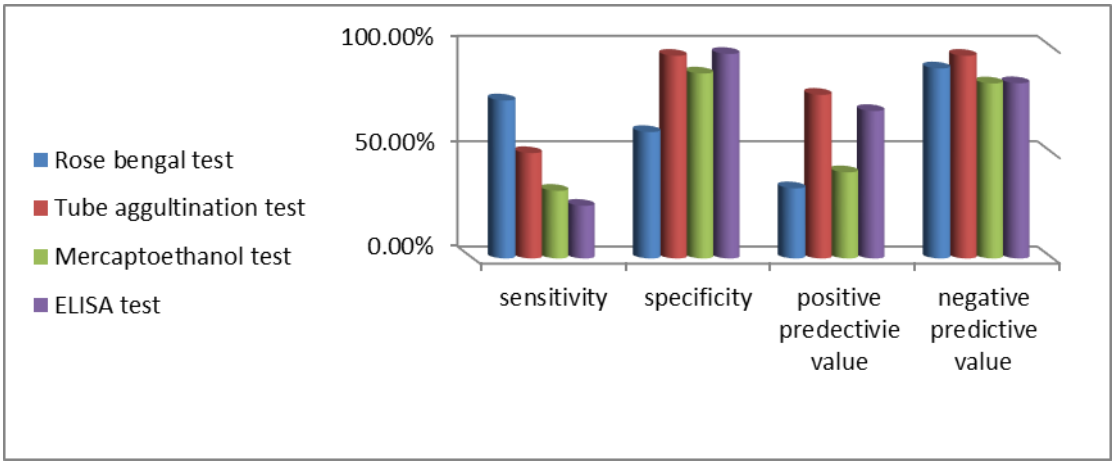

Figure (4): Efficiency of serological tests

The low sensitivity of ELISA test in In comparison with other serological, that's may be due to high number studied cases were aborted and pregnancy buffalo (acute stage of brucellosis) while ELISA kit used in study for detection IgG (chronic infection).

The highly Specificity of ELISA that recorded in current study is agreement with (OIE,2004) which explained ELISA test as a qualitative test and Superior test in Compare it with other serological test.

Conclusions: Buffalos Brucellsis were distribution in Salahaldeen governorate**.**For high sensitivity and high specificity diagnosis, Rose Bengal and ELISA must be use together. Not necessary agreement accrue between serological tests and PCR test.

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