Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 5, Issue, 07, pp.1926-1929, July, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

SEROLOGICAL AND BACTERIOLOGICAL IDENTIFICATION OF *Brucella melitensis* FROM NATURALLY INFECTED SHEEP

[1]Habtamu, T. T. [2]Rathore, R. [3*]Dhama, K. and [4]Karthik, K.

[1]Mekelle University, College of Veterinary Medicine, Mekelle, Ethiopia. P.O. Box: 231
[2,4]Division of Bacteriology and Mycology, [3]Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.)-243122, India

ARTICLE INFO

Article History:
Received 16th April, 2013
Received in revised form
22nd May, 2013
Accepted 03rd June, 2013
Published online 18th July, 2013

Key words:
Brucella melitensis,
Isolation,
Identification,
Sheep.

ABSTRACT

Brucellosis is a notable disease, most widespread bacterial zoonoses that still of veterinarian, public health and economic concern in many developing countries including India. Early detection and segregation of infected animals are important in order to control the disease. The present study was done with the objective of isolation and identification of *B. melitensis* from naturally infected sheep in Sharanpur District, UP (India) using bacteriological and the classical serological techniques; RBPT, STAT, Modified-STAT and i-ELISA. Out of 145 ovine serum samples collected, 48(33.1%) were found to be positive by RBPT taken as a primary screening test. From 48 positive serum samples, 39(81.25%) were found to be positive by STAT, 37(77.08%) by Modified-STAT and 35(72.92%) by i-ELISA. Thus, i-ELISA showed 4 (8.33%) less positive, reduced doubtful by 3 (6.25%) and showed 7 (14.58%) more negative as compared to STAT and 2 (4.16%) less positive, reduced doubtful by 1 (2.08%) and showed 3 (6.25%) more negative to modified-STAT. On the basis of colony morphology, staining characters, phenotypic and biochemical characterizations, the organisms from clinically infected sheep knee joint fluid and aborted foetal samples were isolated and identified as *B. melitensis*. In addition, two isolates of *B. abortus* were also identified from liver sample of aborted bovine foetus.

INTRODUCTION

Brucellosis, caused by members of the genus *Brucella*, is an important re-emerging bacterial zoonosis and a significant cause of reproductive losses in animals. It is still an uncontrolled serious public health problem in many developing countries including India (Saleem *et al*., 2004; Benkirane, 2006; Minas, 2006). The economic losses by brucellosis in animals are due to abortions, premature births, decreased milk production and repeat breeding, and may lead to temporary or permanent infertility in infected livestock (Erdenlig and Sen, 2000; Al-Talafhah *et al*., 2003). Brucellosis due to *Brucella melitensis* is widespread in India and is considered to be the major cause of abortion in small ruminants producing severe economic loss. The disease is usually caused by *Brucella abortus* in cattle, *B. melitensis* or *B. ovis* in small ruminants, *B. suis* in pigs and *B. canis* in dogs (Corbel and Brinley-Morgan, 1984; Anon, 2001). Two potential novel species of marine mammal origin, *Brucella pinnipedialis* (formerly '*Brucella pinnipediae*') and *Brucella ceti* (formerly '*Brucella cetaceae*'), have been proposed recently (Cloeckaert *et al*., 2001; Foster *et al*., 2007). The disease in humans is caused by *B. abortus*, *B. melitensis*, *B. suis* biovars 1-4, and rarely, *B. canis* or marine mammal *Brucella*. Ovine/caprine brucellosis caused by *B. melitensis* is by far the most important clinically apparent disease in humans (Davis, 1990; England *et al*., 2004; Karthik *et al*., 2013). Live vaccines for *B. abortus* and *B. melitensis* are also pathogenic for humans. *B. ovis*, *B. neotomae* and *B. suis* biovar 5 have not been linked to human disease. Man can get infection by direct contact with animals, consumption of unpasteurised milk and other dairy products from infected animals as well as by inhalation of airborne agents.

Corresponding author: K. Dhama
Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.)-243122, India

Due to the high DNA-DNA relatedness (90%) among *Brucella* species, it was suggested that the genus should comprise only one species, *B. melitensis*, with the six biovars Melitensis, Abortus, Canis, Neotomae, Ovis and Suis (Verger *et al*., 1985). However, in 2003, the ICSP Subcommittee on the Taxonomy of *Brucella* agreed unanimously on a return to pre-1986 *Brucella* taxonomy and as a consequence to the reapproval of the six *Brucella* species with the recognized biovars (Osterman and Moriyon, 2006). The different species and biovars of *Brucella* have been characterized according to growth behaviour on different media, CO_2 requirement, H_2S production, growth in the presence of dyes (thionine and basic fuchsin), reaction with monospecific A and M antisera, bacteriophage typing (Alton *et al*., 1988; Corbel, 1991; Muz *et al*., 1999) and light and transmission electron microscopy. Such methods would have required several weeks time to complete (Stemshorn, 1984; Al Dahouk *et al*., 2005; Elfaki *et al*., 2005). In addition, standard serological tests used to detect *Brucella* have also required several weeks time to complete and have not been able to distinguish between species of *Brucella* (Gurturk *et al*., 2000; Ongor *et al*., 2001; Elfaki *et al*., 2005). The methods currently available to identify species of infecting *Brucella* require the isolation of bacteria on selective media followed by quantitative analysis of phenotypic properties of the organism.

MATERIALS AND METHODS

Bacterial Strains

Field isolates of *Brucella* species, reference strain of *B. melitensis* 16M (from National *Brucella* Laboratory, IVRI) and vaccine strain of *B. abortus* S19 (from Division of Biological Products, IVRI) were used in the present study.

Sample Collection

A total of 145 serum samples from female sheep with clinical signs of abortion in Sharanpur District, Uttar Pradesh, India were collected aseptically for the study. Joint fluid samples from clinically infected sheep with swelling knee joint and the aborted foetal materials were collected in a sterile container and in Amies transport medium stored in icepack and transferred to the laboratory.

Bacteriological Examinations

Joint fluid samples were immediately inoculated aseptically to Blood agar and *Brucella* agar without *Brucella* selective supplement. Isolation and identification of *Brucella melitensis* was done as detailed in Bergey's Manual of Systemic Bacteriology (Corbel and Brinley-Morgan, 1984) and OIE (2000). The aborted foetal contents (liver and spleen) were crushed into pieces with sterile mortar and pestle in PBS (pH 7.2) and streaked on to Blood agar and *Brucella* selective agar without *Brucella* selective supplement. The agar plates were incubated at 37°C in an atmosphere of 5-10% CO_2 for 72 hrs. A replica plate was also kept at 37° C without CO_2 tension. Further identification of the organism was done by their morphology and microscopic examination using Hucker's modified Gram stain method (1923) and modified Ziehl-Neelsen stain technique outlined by Stamp *et al* (1950). Biochemical characteristics *viz.* oxidase test (Carter and Cole, 1990), catalase test, urea hydrolysis, nitrate reduction test and hydrogen sulphide (H_2S) production, growth in the presence of thionin and basic fuchsin dyes at three different concentrations (10-40 µg/ml) (Corbel and Brinley-Morgan, 1984) were carried out.

Serology

Serum samples collected from 145 sheep suspected for *Brucella* infection were analyzed by classical serological tests such as Rose Bengal plate test (RBPT), standard/modified tube agglutination tests (STAT/Modified-STAT) and indirect enzyme linked immune sorbent assay (ELISA) following the standard protocols. RBPT was done according to the procedure described by Alton *et al*. (1975) with Rose Bengal plain antigen procured from Division of Biological Product, IVRI, Izatnagar. About 30µl of RBPT plain antigen and equal volume of test serum were taken on a grease free clean glass plate, and mixed by glass rod or wood stick which was then followed by swirling. Any degree of agglutination within 3-4 minutes was taken as positive. *Brucella* negative and positive sera were taken as negative and positive control, respectively. Antigen and serum samples were brought to room temperature prior to performing the test. Out of 145 serum samples collected previously, those sera that gave positive reaction with RBPT were further subjected to the standard tube agglutination test (STAT) using the method described by Stem shorn *et al.* (1984), and modified tube agglutination test (OIE, 1996).

The modified tube agglutination test was performed in clear glass or plastic tubes of approximately 5 ml total volume by placing 0.8 ml of phosphate buffer saline solution (PBSS) with 10 mM EDTA having pH 7.2, into the first tube and 0.5 ml volumes of PBSS in the remaining tubes of a series of at least five tubes per each serum sample under test. A 0.2 ml serum was added to the first tube, mixed and then 0.5 ml was transferred to the next tube. A further volume of 0.5 ml was transferred to subsequent tubes and in the last tube 0.5 ml of serum dilution was discarded to give a series of doubling dilutions. An equal volume (0.5 ml) of Plain antigen of *Brucella abortus* S99 (IVRI, Izatnagar) was then added to each tube, and the tubes were incubated at 37°C for 24 hours. In standard tube agglutination test, phenol saline (pH 7.2) was used in place of phosphate buffer saline solution. The results were compared with the antigen control tube showing 50% agglutination and in both the cases; agglutination was determined by reading the degree of clearing and sedimentation of the tubes. A titre of 1:40 (i.e. 50% agglutination at 1:40) or more was indicative of infection, whereas 50% or above reaction in titre of 1:20 was considered a suspicious and a titre of 1:10 was treated as negative. Screening of serum samples positive to RBPT was again rolled out by using commercially available ELISA kits following instructions described by manufacturer (Institut Pourquir, France). Briefly, 190 µl serum dilution buffer was added to each 96 well of *Brucella* LPS antigen pre-coated ELISA microplates and 10 µl of serum sample (1:20) per well was added leaving the first three wells; one for negative control and the other two for positive controls. The contents were then homogenized by gentle shaking and incubated overnight at 4oC. After washing three times with 1x wash solution (20x), 100µl of a peroxidase conjugate anti-ruminant IgG monoclonal antibody diluted (1:100) with dilution buffer1 (Conjugate dilution buffer) was added to each well and incubated at room temperature for 30 minutes followed by washing three times as described above. A ready to use substrate solution (TMB) in a volume of 100 µl was added per well as an indicator and incubated at room temperature in dark for 20 minutes. To stop the reaction, 100 µl of stop solution (0.5M H_2SO4) was added to each well and the absorbance at 450nm was determined with an ELISA reader (Dynatech Laboratories). The result was interpreted by its S/P%:

$$S/P\% = \frac{100 \times (\text{OD450 value of sample} - \text{OD450 value of negative control})}{(\text{Mean OD450 value of positive controls} - \text{OD450 value of negative control})}$$

The interpretation of the result will be valid provided that OD_{450} value of positive control is greater than or equal to 0.600, and the ratio between the mean OD_{450} value of positive controls to OD_{450} value of negative control is greater than or equal to 3. Any sample with S/P% value equal to or lower than 110% were considered to be from animals which do not have any specific antibodies to the *Brucella* LPS antigen where as S/P% value between 110% and 120% were considered to be doubtful and samples with equal or higher than 120% were from animals having specific antibodies to the *Brucella* LPS antigen.

RESULTS AND DISCUSSION

Field isolates of *Brucella* species have been maintained in serum dextrose agar and *Brucella* selective medium to isolate and identify *B. melitensis*. A characteristic of *Brucella* growth with very small, glistening, smooth, round and pin-point like colonies with honey like appearance were observed on blood and *Brucella* selective agar plates after 72 hrs incubation at 37°C. Similar observations were also recorded by Corbel and Morgan (1984). The organisms did not grow on MacConkey agar, Muller Hinton agar and found to be non-hemolytic on blood agar. These exclude rapidly growing, haemolytic or lactose fermenting organisms. Microscopic examination of Gram stained cultures revealed small Gram negative coccobacilli arranged singly, in pairs, or in groups and on Modified Ziehl-Neelsen (MZN) stain, the *Brucella* organisms were stained red against a blue background. On different biochemical reactions, *Brucella* organisms were found to be positive for catalase, oxidase, urea hydrolysis and nitrate reduction tests and negative for indole production, citrate utilization, Methyl red and Voges-Proskauer tests (Table 1). Similar findings were reported in different isolates of *Brucella* species by Koneman *et al.* (1997) and Erdenlig and Sen (2000). On the basis of colony morphology, staining characters, and biochemical tests, the organisms were identified as *Brucella* species. The isolates have been further differentiated phenotypically into species and partially to biovars using parameters such as CO_2 requirement, H_2S production, and growth on media plates containing thionin and basic fuchsin dyes at three different concentrations. Accordingly, *Brucella* species grown on tryptic soya agar media containing both thionin and basic fuchsin dyes at concentration of 10 µg/ml, 20 µg/ml, and 40 µg/ml have been taken as *Brucella melitensis* where as isolates with no growth at all concentrations in both the cases (thionin and basic fuchsin) were considered as *Brucella abortus* biovar 2 and those grow on media with thionin at only 40 µg/ml (1:25,000) concentration and basic fuchsin at all concentrations have been considered as *Brucella*

Table 1. Result summary on biochemical characteristics of *Brucella* species

Biochemical profile	*Brucella abortus* 1	*Brucella abortus* 2	*Brucella melitensis*
Hemolysis (blood agar)	-	-	-
Acid production	-	-	-
Urea hydrolysis	+	+	+
Nitrate reduction	+	+	+
Citrate utilization	-	-	-
Indole production	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Methyl Red	-	-	-
Voges-Proskauer	-	-	-

abortus biovar 3. Requirement of CO_2 has been observed in the two biovars of *Brucella abortus*, but not in *B. melitensis* (Table 2). This was in agreement with the reports by Morgan (1961), Corbel (1991), Anon (2001), and Stack and MacMillan (2003). Abscence of growth on media containing streptomycin (2.5µg/ml) and requirement of CO_2 by field isolates of *B. abortus* differentiated the organisms from vaccine strains *B. melitensis* Rev1 and *B. abortus* S19, repectively.

Table 2. Differentiation of the species of the *Brucella* isolates

Characteristics	*B. melitensis*	*B. abortus* isolate 1	*B. abortus* isolate 2
H_2S production			
1st Day	-	+	++
2nd "	-	++	++
3rd "	-	++	+++
4th "	-	+++	++++
CO_2 requirement	-	+	+
Growth on media containing:			
Thionin*			
1:25,000	+	–	+
1:50,000	+	–	–
1:100,000	+	–	–
Basic fuchsin*			
1:25,000	+	–	+
1:50,000	+	–	+
1:100,000	+	–	+

*dye concentration, 40µg/ml (1:25,000), 20µg/ml (1:50,000), and 10µg/ml (1:100,000) in tryptic soya agar.

Besides the bacteriological identification, a cross confirmation of the genus *Brucella* has been done on serum samples collected from sheep by the classical serological techniques. RBPT was taken as a primary screening test for the downstream comparison between STAT/Modified-STAT, and i-ELISA. From a total of 145 ovine serum samples screened for presence of *Brucella* antibodies, 48 of them were found to be positive by RBPT. From 48 RBPT positive serum samples, 39 (81.25%) were recorded as positive, 6 (12.50%) doubtful and 3 (6.25%) negative by STAT as compared to the modified-STAT where 37 (77.08%) serum samples were positive, 4 (8.33%) were doubtful and 7 (14.58%) were negative. Further analysis by i-ELISA revealed that 35 (72.92%) serum samples were positive, 3 (6.25%) were doubtful and 10 (20.83%) were negative. Thus, i-ELISA showed 4 (8.33%) less positive, reduced doubtful by 3 (6.25%) and showed 7 (14.58%) more negative as compared to SAT and 2 (4.16%) less positive, reduced doubtful by 1 (2.08%) and showed 3 (6.25%) more negative to modified-STAT. Screening of brucellosis or detection of *Brucella* antibodies using i-ELISA revealed relatively better sensitivity and specificity than STAT and modified-STAT (Table 3). A similar comparative result has been reported by Nasir *et al*. (2005). Respectively, the field isolates were identified and identified as *B. abortus* isolate 1, *B. abortus* isolate 2 and *B. melitensis* based on biochemical profiles, cultural characteristics, and partial bacteriological bio typing techniques. In conclusion, *B. melitensis* is the main aetiologic agent of brucellosis in small ruminants. Ewes' and nanny-goats' aborted foetuses and products derived from sheep and goats remain the main source of infections to man.

Table 3. Comparison of STAT, Modified STAT and ELISA

Tests	No. of samples	Antibody titers		
		1:10 (negative) (≤ 110%)	1:20 (doughtful) (110 - 120%)	1:40 or more (positive) (≥ 120%)
STAT	48	3 (6.25%)	6(12.50%)	39(81.25%)
Modified-STAT*	48	7(14.58%)	4(8.33%)	37(77.08%)
i-ELISA	48	10(20.83%)	3(6.25%)	35(72.92%)

* Interpretation of STAT and modified-STAT result is same with only difference in serum dilution buffer

The studies in various parts of India indicate that the disease is widespread among sheep populations. Most surveys of brucellosis in India rely on serological test only, without isolation of *Brucella* sp. and this can be misleading for the ultimate action to be taken. Confirmatory diagnosis must be provided by the isolation of aetiological agents. Therefore, the isolation of *B. melitensis* is important to study the epidemiology of the disease in the country. The detection of antibodies in 48 serum samples and the isolation of B. *melitensis* from aborted sheep foetus and blood samples revealed its predominant cause of the infection in this region (Sharanpur District, UP) and due to that, the disease may threat human and animal health. Brucellosis may be acquired directly through contact with contaminated material or aerosol infection or indirectly by grazing on contaminated pastures or through other materials. The traditional grazing management system of sheep and goats is still in use in this district. Moreover, several flocks belonging to different owners may graze the same pasture on the same day or the following day(s), which may spread the infection directly among herds. *B. melitensis* vaccine strain Rev 1 sometimes isolated from some sheep abortions and may lead to misdiagnosis of the disease. However, the isolates in the present study were found to be different from *B. melitensis* Rev 1 and this finding may suggest that this vaccine strain is not the causative agent of ovine brucellosis in this region. The isolation of the *B. melitensis* from aborted sheep foetuses in the present study may show the importance of this agent in aetiology of ovine brucellosis and abortions in sheep. Similar findings of *B. melitensis* in sheep flock have been reported by Vivekananda *et al.* (2012).

Acknowledgements

The authors are grateful to Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out this study. This work was fully financed by the Ethiopian Government, Ministry of Education in collaboration with Indian Government, IVRI.

REFERENCES

Al Dahouk, S., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W.D., Scholz, H.C. and Neubauer, H. 2005. Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Crit. Rev. Microbiol., 31(4): 191-196.

Al-Talafhah, A.H., Lap, S.Q. and Al- Tarazi, Y. 2003. Epidemiology of ovine brucellosis in Awassi sheep in Northern Jordan. Prev. Vet. Med., 60: 297-306.

Alton, G.G., Jones, L.M. and Peitz, D.D. 1975. Laboratory Techniques in brucellosis. IInd. WHO Monograph Series 55: WHO, Geneva.

Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. 1988. Techniques for the Brucellosis Laboratory (Techniques et Pratiques). Paris: INRA.

Anon. 2001. Brucellosis in sheep and goats (*Brucella melitensis*). Scientific Committee on Animal Health and Animal Welfare. European Commission. http://ec.europa.eu/food/fs/sc/scah/out59_en.pdf

Benkirane, A. 2006. Ovine and caprine brucellosis: World distribution and control/eradication strategies in West Asia/North Africa region. Small Rum. Res., 62: 19-25.

Carter, G.R. and Cole, J.R. 1990. Diagnostic procedures in veterinary Bacteriology and Mycology. 5th Edn. Academic press. Inc. California. Pp. 522.

Cloeckaert, A., Verger, J.M., Grayon, M., Paquet, J.Y., Garin-Bastuji, B., Foster, G. and Godfroid, J. 2001. Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the *omp2* locus. Microbes Infect., 3: 729–738.

Corbel, M.J. and Brinley-Morgan, W.J. 1984. Genus *Brucella*. In Bergey's Manual of Systematic Bacteriology, vol. 1, Pp. 377–388. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Corbel, M.J. 1991. Identification of dye-sensitive strains of *Brucella melitensis*. J. Clin. Microbiol., 29: 1066-1068.

Davis, D.S. 1990. Brucellosis in wildlife. In: Nielsen K., and Duncan J.R. (Eds.), Animal brucellosis, CRC Press, Boca Raton. Brucellosis: a re-emerging zoonosis 325. Pp. 321–334.

Elfaki, M.G., Al-Hokail, A.A., Nakeeb, S.M. and Al-Rabiah, F.A. 2005. Evaluation of culture, tube agglutination, and PCR methods for the diagnosis of brucellosis in humans. Med. Sci. Monit., 11(11): 69-74.

England, T., Kelly, L., Jones, R., MacMillan, A. and Wooldridge, M. 2004. A simulation model of brucellosis spread in British cattle under several testing regimes. Prevent. Vet. Med., 63: 63–73.

Erdenlig, S. and Sen, A. 2000. Isolation and biotyping of *Brucella* species in aborted sheep fetuses. Pendik. Vet. Microbiol., 31: 31-42.

Foster, G., Osterman, B., Godfroid, J., Jacques, I. and Cloeckaert, A. 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int. J. Syst. Evol. Microbiol., 57: 2688–2693.

Gurturk, K., Solmaz, H., Ekin, I.H., Aksakal, A. and Gulhan, T. 2000. Bacteriological and serological examinations of aborting sheep in Van Region. Univ Yuzuncu Yıl. J. Fac. Vet. Med., 11: 19-22.

Hucker, G.J. and Conn, H.J. 1923. Methods of Gram Staining. Tech Bull of NY State Agric Exp No 102.

Karthik, K., Rathore, R., Verma, A.K., Tiwari, R., Mahima. and Dhama, K. 2013. Brucellosis – still it stings? *Livestock Technology*, March issue (In Press).

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C. and Winn, W.C. 1997. *Brucella* species. In: diagnostic Microbiology, 5th ed. Lippincott, Philadelphia, USA. Pp. 431-436.

Minas, A. 2006. Control and eradication of brucellosis in small ruminants. Small Rum. Res., 62: 101-107.

Morgan, W.J.B. 1961. The Use of the Thionin Blue Sensitivity Test in the Examination of *Brucella*. Central Veterinary Laboratory, Weybridge, Surrey. J. Gen. Microbiol., 25: 135-139.

Muz, A., Ertaş, H.B., Ongor, H., Gulcu, H.B., Ozer, H., Eroksuz, H., Dabak, M. and Kalender, H. 1999. Bacteriological, serological, and pathologic studies on abortus cases of goats and sheep in Elazig and its vicinity. Tr. J. Vet. Animal. Sci., 23: 177-188.

Nasir, A.A., Perveen, Z. and Ikram-ul-Haq, M. 2005. Comparative study of standard and modified serum agglutination tests for the diagnosis of brucellosis in animals. Pakistan J. vet., 25 (1): 33-34

OIE, 1996. Manual of standards for diagnostic tests and vaccines 3rd Ed., Office International des Epizooties, Paris, France. Pp: 251.

OIE, 2000. Manual of Standards for Diagnostic Tests and Vaccines. Caprine and ovine brucellosis. OIE., Paris. Pp. 475-489.

Ongor, H., Muz, A. and Cetinkaya, B. 2001. Comparison of ELISA with other serological tests in the diagnosis of ovine brucellosis. Turk. J. Vet. Sci., 25: 21-26.

Osterman, B. and Moriyon, I. 2006. International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Brucella*. Minutes of the meeting, 17 September 2003, Pamplona, Spain. Int. J. Syst. Evol. Microbiol., 56: 1173–1175.

Saleem, A.N., Rhaymah, M.S. and Shamoon G.N. 2004. Isolation and seroprevalence of ovine brucellosis. Iraqi. J. Vet. Sci., 18: 31-38.

Stack, J.A. and MacMillan, A.P. 2003. Identification and Biotyping of *Brucella* spp. FAO/WHO Collaborating Centre for Reference and Research on Brucellosis. Central Veterinary Laboratory. www.fishery.moag.gov.il/NR/rdonlyres/F0681F75-0AF2.../webtype3.doc

Stamp, J.T, McGiven, A.D., Watt, J.A.A. and Nisbet, D.I. 1950. Enzootic abortion in ewes. Transmission of the disease. Vet. Rec., 62: 251-254.

Stemshorn, B.W. 1984. Recent progress in the diagnosis of brucellosis. Dev. Biol. Stand. 56: 325-340.

Verger, J.M., Grimont, F., Grimont, P.A.D. and Grayon, M. 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol., 35: 292–295.

Vivekananda., Shome, R., Isloor, S., Rathnamma., Veeregowda, B.M., Suryanarayana, V.V.S., Shome, B.R. and Prabhudas, K. 2012. A multi-pronged approach for diagnosis of *Brucella melitensis* infection in sheep flocks. Indian J. Anim. Sci., 82 (2): 135–138.

****