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INVESTIGATION OF THE PERFORMANCE OF BIPHASIC MEDIA IN THE CULTURE OF BRUCELLA

Noha A. Helmy* and Sally M. Abd Elsalam

Bacterial Sera and Antigens Research Departement, Veterinary Serum and Vaccine Research Institute, Cairo, Egypt.

***Corresponding Author: Noha A. Helmy**
Bacterial Sera and Antigens Research Departement, Veterinary Serum and Vaccine Research Institute, Cairo, Egypt.

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ABSTRACT
Brucellosis is a common and significant bacterial disease among the world. Infected animals are generally detected by serological tests. Brucella culture and identification provide a definitive diagnosis but it needs several days. The aim of this study is to propose a biphasic media to cultivate Br. abortus S99for diagnostic and research purposes and to compare it with the glycerol potato agar OIE medium that is frequently used in routine preparing antigens and to assess the effectiveness in culturing Br. abortus S99. The culture obtained from biphasic media was evaluated microscopically, biochemically and molecularly .Rose Bengal antigens were prepared and compared with the one prepared by classical culture of Br. Abortus S99. All sera samples were tested by plate for all antigens prepared. The three antigens were prepared from biphasic media had the agreement with classical Rose Bengal and SAT. The biphasic media exhibited good growth of the strain in less time. The present study depicts the facilitate manipulation, low cost effective that help in cultivation of brucella without doing subculture and may use with limited expertise or equipments if all safety precautions are taken.

INTRODUCTION
Brucellosis is a major zoonotic disease that occurs in domestic animals and wildlife as well as humans worldwide[1] especially in Mediterranean countries[2], the Middle East, India and South America constituting an important public health problem and remains an uncontrolled problem.[3,4] Although brucellosis is rarely life-threatening, it can be a severely debilitating and disabling disease and is regaining importance because of the increasing threat of its use as a biowarfare agent and its re-emergence in areas where it was thought to be nearly eradicated.[5] The disease is a major cause of direct economic losses due to abortions, stillbirths, weight loss, decreased milk production and is an impediment to trade and export.[6,7] In addition, the WHO laboratory biosecurity manual describes Brucella organisms as belonging to risk group3 microorganisms.[8] The genus Brucella consists of 11 species, and their classifications are mainly based on biological and biochemical characteristics and host preferences.[9] The main pathogenic species worldwide are B. melitensis, B.abortus and B.suis. Animals and food products are the primary source of human infection where the mucosal routes are the most common modes of transmission.[10] Brucellosis also called Job-related disease because vets, farmers and workers exposed to infected materials or diseased animals[11] and remains a significant cause of laboratory acquired infection.[12] Brucellosis not only severely harms human health but also strongly influences the development of animal husbandry and there is variation in the clinical manifestation between species which are non specific, protean and its diagnosis is very difficult. Hence, laboratory diagnosis is of paramount importance for confirming brucellosis. Serological tests remain the mainstay of laboratory diagnosis. Rose Bengal plate test (RBPT) and standard agglutination tube (SAT) are the most popular serological test used to detect and eradicate brucella[13,14] sometime may be supplanted by complement fixation test (CFT). The antigens used in these tests should be derived from smooth cells so the choice is on B.abortus S99 which is of greatly reduced virulence and pathogenicity,CO2 independent beside is dominant A antigen[15] which comply with minimum standard requirements for serological reagents. For antigen production, the strain must be pure cultures to produce a suspension of whole smooth cells are able to detect brucella agglutinin rather than rough cells which increase the opportunity of cross reaction with other organisms. So it is very important for B.abortus S99 to grow in artificial culture media provide it all the nutritional components besides optimum environment to obtain it with the characters mentioned. Successful culture necessitates a careful selection of the best suitable culture method. Bacterial culture media can be classified according its nutritional components, functional use or consistency which be either liquid, semi solid or solid forms.[16] Brucella strains are relatively slow growing, may undergo changes in colonial morphology accompanied by alterations in antigenic structure, phage susceptibility and virulence i.e.

dissociation when are grown in liquid media.[17]To overcome this problem, automated liquid culture systems or fermentors are used however, they are expensive, out of reach financially for laboratories in developing countries, difficult to operate and need more skills to produce perfect cells[18] In contrast, culturing on solid media is a convenient way and nearly the method of choice to obtain uniform brucella cells but it has some drawbacks for biomass production like subcultures which have been advocated to maximize the low concentration of brucella cells obtained which may in turn increase the opportunity of contamination beside time consuming and labor intensive. Many methods have been developed for culturing brucella species from specimen, including Castaneda method, lysis concentration, automated systems and clot culture[19]

In the forties and fifties, Hestrin[20] mentioned the use aqueous sucrose over a nutrient agar for the production bacterial levansucrase, Tyrrell[21] stated that combination of a layer of solid nutrient medium overlaid with nutrient broth have produced concentrated bacterial population 2 to 30 fold over broth control. Castaneda[22] described a double or biphasic medium bottle containing broth and agar slant for the isolation of brucella from blood followed by Scott who described a practical technique for bacterial blood culture but the biphasic medium has received scant attention in the literature and no careful evaluation in parallel with conventional blood culture media In the recent, modifications to Castaneda method have been developed for the routine diagnosis of bacteremia to isolate fastidious organisms[23,24] in addition to several commercial products were available followed same architectural design have been recommended for the recovery of brucella[25] due to its easiness of manipulation and might have an impact on the yield of the bacteria.

Hence, for this reasons, this study was carried out to determine the utility of the simplified Biphasic culture technique to produce large number of B.abortus S99 cells to evaluate it against the conventional culture technique and to know its impact.

MATERIAL AND METHODS

Media formulations

Biphasic media (BM) consists of the solid sheet and the overlay liquid culture media. The solid phase consists of the following ingredients glycerol, potatoes, peptone, meat extract, sodium chloride, agar and completion with distilled water until 1000 ml. The mixture was homogenized then placed in sterile Roux bottles and autoclaved for 20 min at 120˚C and 1.5 lb. The bottles were put flat side down on a bench overnight for solidification of the agar then incubated at 37 C for two days to control sterility. For the overlay, tryptic soy broth was prepared and autoclaved for 15 min. When the media cooled down was added aseptically to the glycerol potato infusion agar bottles GPIAM and reincubated to check sterility.

Bacterial Strain and Growth Conditions

Freeze dried vials of B.abortus S99 seed culture was kindly provided from our brucella Vetrinary Sera and Vaccines Research Institute(VSVRI) and reconstituted with 2ml sterile double distilled water DDW. Suspensions were grown in tryptose soy agar (Oxoid) at 37 C for 72 hours then harvested in tryptose soy broth glycerdized and stored at -20 for future use.

At the moment of use, the frozen cultures were diluted in 0.85%saline solution, and read in spectrophotometer at 640 nm. Glycerol potato infusion agar media (GPIAM) which performed as described by OIE method[26] served as control for the evaluation the biphasic culture method used for growth of B. abortus.

CULTIVATION

The overlay broth of BM was inoculated with 1 ml culture of B.abortusS99 adjusted in parallel with GPIAM (OIE).[26] They were incubated at 37 C and the biphasic bottles stand in vertical position and the cultures were monitered for growth through incubation period .Sub culturing occurred by allowing the broth to flow over the solid phase on of the Roux bottles at various time intervals (8, 16 ,24 hours). The cultured broth remained on the solid phase for 30 minutes then inverted and incubated at 37C.The bottles were checked and observed for any changes and the day of appearance of the colonies was recorded. For the GPIAM bottles, first B.abortus S99was cultivated on slopes of the same media incubated at 37Cfor 48 hours then dissolved with phosphate buffer saline PBS, transferred to the Roux bottles, spread it uniformly over agar surface. Roux bottles were inverted, incubated at 37 C for 48 to 72 hrs. The performance of the biphasic media was compared to that of the GPIAM. The brucella colonies were examined for sterility then characterized initially by Gram stain, microscopy and colony morphology followed by biochemical tests like urease, H2S production, sensitivity to dyes such as basic fuchsin (1:500000 and 1:100000) and the acriflavine was used to estimate colonial roughness. The growth and the degree of contamination was observed for each culture. Serological confirmation of the colonies was done by performing slide agglutination test using B.abortus mono specific A and M antisera and molecular methods.

DNA Preparation and Molecular characterization

The genomic DNA of the Brucella abortus S99 cultures was extracted using Qia AmpDNA extraction kit(Qiagen) according to manufacturer's instructions and was stored at -20 C.Multiple Locus variable number tandem repeat analysis MLVAtyping assay consist of the primer sets as described by[27] were performed for confirmation of the brucella growth in biphasic media. PCR amplification was performed in a total volume of 50 ul containing 2 ul of template DNA,20 pmol of each primers,25 ul of master mix (Qiagen) and D.D.W.

Cycling profile for amplification was starting one cycle at 96 C for 5 minutes followed by 30 cycles at 96C for 30 sec, 60˚C for 30 sec and 70˚c for 60 sec and the final step 70 C for 5 minutes. Following PCR reaction,15 ul of the PCR products were mixed with 3 ul of loading buffer and were run in 3%agarose gel electrophoresed in Tris acetate EDTA (TAE) at low volt and the amplified DNA bands were visualized in ethidium bromide staining under UV light . 100 bp DNA ladder (Qiagen) was used as marker.

SDS- PAGE for LipoPoly Saccharides LPS

LPS was extracted by according to[28] and estimated by the method of[29] SDS-PAGE was performed with 14% and stained with silver stain procedures.

Preparation of Rose Bengal Plate Test Antigens {RBPT}

RBPT antigens were prepared from Br. Abortus S99 from BM according to the technique of OIE[26] using the recommended chemicals and equipments. Then the antigens were tested compared for their efficiency using the standard RBPT antigen prepared from Br. abortus S99 produced by Veterinary Sera and Vaccines Research Institute,Cairo Egypt .SAT antigen was also provided by VSVRI.

Serum Samples

Sera before and after vaccination of cattles with Br. abortusS19 as well as infected one were used in the experiment. The study also included positive and negative sera samples as control.

Serological Methods

All sera samples were first examined by standard tube agglutination test(SAT) beside RBPT performed according to standard procedures.[26] Described by OIE[26], SAT was performed by preparing two –fold serial dilutions of the sera sample starting at a dilution of 1:20 in a microtitre plate and the addition of an equal volume of brucella antigens. They were incubated for 24 hours at 37 C. SAT results were evaluated qualitatively.

Briefly, for the RBPT, sera samples (30ul) were mixed thoroughly with an equal volume of Rose Bengal antigens on a white porcelain plate which was rocked slowly and observed. Results were evaluated as negative when agglutination was absent and scored from 1+ to 3+ positive according agglutination strength.

Data were subjected to one way analyais of variance ANOVA and the post- hoe test was applied.

RESULTS

The objective of this investigation was to demonstrate the improvent of brucella cultivation on the BM. Analysis of the results was limited to the two GPIAM and BM. BM constitute of ingredients that were available in microbiology laboratory inexpensive and easy in preparation. The growth of B. abortus S99 was observed in all bottles of BM .There was early growth of the strain on the BM unlike monophasic GPIAM.

Cultures were easily seen after 24 hours of incubation on the solid phase of BM regarding the duration of incubation in liquid phase which showed turbidity at 8 hours and were inverted at 8,16 and 24 hours for incubation on solid phase. While it took 3 to 5 days for the growth of colonies on the mono phasic media with no big differences between the both media. The macroscopic appearance of the BM culture changed considerably during bacterial growth. Till the first16 hours, growth appeared as relatively homogenous suspension in the liquid phase. There after the cells started to aggregated forming like a mat in 10% of 24 hour incubation bottles. 8 and16 hours liquid phase incubation did not show any contamination in the culture medium while the difference between the number of contaminated cultures on GPIAM and in 24 hours liquid phase incubation BM was (4.%) and (5%) respectively. The sterile culture bottles were selected and compared in both types of culture media were not found to be of significant difference.

The efficiency of biphasic method to obtain B.abortusS99 mass was satisfactory and when they exhibited to biotyping assays they showed typical characteristics of B.abortus (Table 1),they were analyzed by agglutination test with mono specific sera(Mono specific antisera A,M: smooth R; rough), growth on dyes (basic fuchsin +) and roughness by acriflavin (table 1).

Table 1: Biochemical profile of B.abortus S99 in different times on BM culture and OIE media and their serological c+haracteristics.

B.abortus S99	H_2S	Oxidase	Catalase	Urease	Agglutination with monospecific sera			Growth on dyes		Roughness
					A	M	R	Thionine	Basic fuchsin	
GPIAM Media	+	+	+	+	+	-	-	-	+	-
Incubation 8 hours	+	+	+	+	+	-	-	-	+	-
Incubation 16 hours	+	+	+	+	+	-	-	-	+	-
Incubation 24 hours	+	+	+	+	+	-	-	-	+	- f

f :few - A; M; R:

Molecular characterization have been carried out to detect any genetical changes in strains cultured by BM. MLVA-3 assay was introduced as a molecular typing tool for bacteria which amplify 3 loci (Table 2) that were useful for species identifications, In the MLVA data using three brucella markers 11,42 and 55, B.aborus S99

cultivated on GPIMA and the one incubated 8,16 hours in liquid phase showed 3 amplicons sized 160,310 and 380 bp while that of 24 hours incubation in liquid phase showed 2 amplicons 160,310 pb.

Table (2): Results for Molecular characterization by BM. MLVA-3 assay for Brucella abortus S99 with two different culture media.

Species	Fragment size (bp)
B.aborus S99 cultivated on GPMA (Control)	160 bp 310 bp 380 bp
B.aborus S99 cultivated on BM and incubated 8 and 16 hours in liquid phase	160 bp 310 bp 380 bp
B.aborus S99 cultivated on BM and the incubated 24 hours in liquid phase	160bp 310 bp

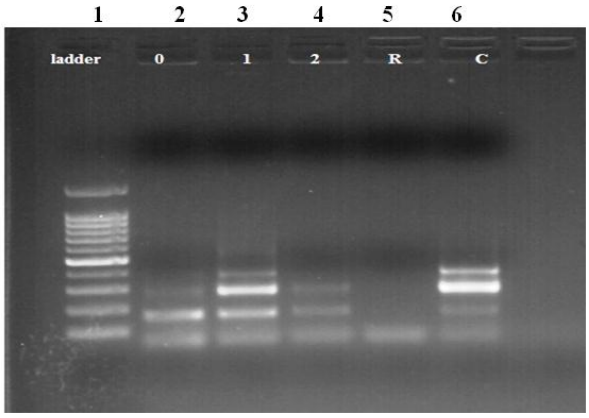

Figure 1 PCR results of the Brucella abortus S99 cultures, Lane 1:100bp DNA marker, Lane 2: 24 hours culture, Lane 3: 16 hours culture, Lane 4: 8 hours culture, Lane 5: -ve control, Lane 6: OIE media (as control).

All SAT positive serum samples were examined by 3 prepared RBPT antigens and were compared with the standard one. RBPT results were evaluated by the degree of agglutination(+++,++,+, and -,)are shown in (Table 3).Two of the prepared RBPT antigens were in complete agreement with the standard one.

Table 3: Serological test results and comparison of the serum samples with 4 different rose Bengal antigens.

Examined antigens	Degree of RBPT agglutination				
	+++	++	+	-	Seropositvity
Control GPIAM (A1)	50	40	30	30	90%
Incubation 8hrs BM (A2)	50	38	32	30	90%
Incubation 16hrs BM (A3)	50	40	30	30	90%
Incubation 24hrs BM (A4)	46	44	20	30	87%

DISCUSSION

Culture media have a key role in growing pathogens, There are various reasons to grow bacteria in the laboratory, one of the this reasons is its utility in diagnosing infectious disease also as initial step in studying its morphology and its identification. When bacteria were cultured it is very important to provide it similar environmental and nutritional conditions that exist in its natural habitat. This will be the media which could be liquid, semisolid or solid and biphasic according the needs of microorganisms. Food requirements of pathogenic bacteria vary widely from organism to organism, some pathogens can easily grow under wide range of nutrition and environment whereas others require stricted nutritional, environmental and certain growth factors and Brucella strain is one of them .It is fastidious, grows poorly on ordinary media and has a little or no fermentative power[30], its growth is slow, scanty, and enriched media is needed to support adequate growth. Bacteria have to be cultured to obtain antigens for developing serological assays or vaccines production. Culture of brucella are usually performed on solid media. However, the use of liquid media is recommended for enrichement and volumizing but brucellae tend to dissociate in broth medium.[26] To obtain antigens for developing serological assays, produced brucella cells should be in adquate quantity, viable at minimal passage. Current cultivation technique which depends on subculture exposed the strain to some changes in its character in addition to contamination. With these points in mind, This study have been planned to asses the efficiency of biphasic culture technique to increase the probability of success brucella culture and produce large amount of B. abortus S99 directly for preparing RBPT antigens from them and compare with the conventional RBPT. Published trials of the biphasic culture system reported its reliability for isolation brucella from blood[31] and proved to better for detecting it than other culture system. It has been found that culturing on biphasing media increases detection (+ culture) by 64.8% compared to isolation on solid media.[32] By using BM containing agar base and broth overlay which was developed by Castaneda in late forties, B abortus S99 grew uniformly and faster than they did on the commonly agar solidified media, Glycerol potato agar was used as the solid phase with Tryptic soy broth TSB broth as a liquid on it. According to the growth results. They grew a substantially greater amount of brucella

strain of lower contamination than the conventional GPIAM, thus this method reduces the possibilities of contamination as well as reduces the risk of laboratory acquired infection and obviate the need for frequent opening of the culture bottle and the necessity of making repeat subculture.

The genus Brucella needs long incubation period to grow[33], GPIAM requires from 3 to5 days to produce B.abortus S99 (OIE) while BM produced visible growth and reduced time to 48 hours including different incubation period in liquid phase from 8to 24 hours this was because there was no need for subculture for mass cultivation, in addition, BM media combines the characteristics of liquid and solid culture media resulting in changing in composition of both phase and the manner in which they are set up which may provide more nutrition that could be conductive to the growth of brucella[34] for large scale. B. abortus colonies were examined classical morphology, biotyping, serology and molecular biology.

Biological characterstics of the Brucella strain may explain the observed discrepancies in the performance of the method. No apparent morphologically variations in the cells between both media except 10% of the bottle of 24 hours incubation in liquid phase have shown some abnormalities of cells on solid phase due to overgrown, while all other cultures were in agreement in biotyping and in agglutination with the one cultivated by OIE method serological identification using mono A,M and R antisera confirming its identity. The LPS of B. abortusS 99 from both cultures were extracted and analyzed 12% SDS-PAGE and stained by silver stain technique resulting similar patterns and is in agreement with values obtained previously.[35,36] PCR is one of the most common diagnostic tool to detect, identify and differentiate species and strains of Brucella.[37] Correct species and biovars demarcation can be accomplished by MLVA-3 assay[38] Molecular identification (PCR) was performed to estimate the genetic characters of the B.abortusS99 obtained from BM and to detect any variations occurred in the cultures. B.abortusS99 from OIE media were amplified 3 loci with a single band of 310,285 and 160 bp respectively by primers described by.[26] The obtained data declared that 8and 16 hours incubation in liquid phase shared the same bands and identical to that of OIE media while 310 bp has been faint little bit with that of 8 hours incubation, while 24 hours exhibited deletion of 380 bp band. Despite this differences genotypically of 24 hours from the control one it is not associated with their antigenicity .Rose Bengal test is used for the diagnosis of brucellosis, its prevalence[15] and as a screening test.[14] DifferentRose Bengal antigens were prepared from the different culture ofB.abortusS99 incubation at 8,16 and 24 hours respctively by OIE method. Grown on BM and standardized according.[25] using standard Rose Bengal antigen, and evaluated according to WHO before using in the evaluation of sera. They were equal to that of standard one. In this study a total of 150 positive (vaccinated and infected) and negative sera samples were tested by the three BM antigens prepared and evaluated with the conventional antigen. The 1st and 2nd antigens was found to be more suitable as they had maximum agreement with the conventional RBPT it can be seen they gave the highest positivity 90% and similarity only to the first antigen followed by the 3rd antigen with less agreement giving 87% This is in disagreement observed with that of 24 hours liquid phase incubation may be explained as Allan et al[39] who mentioned that the antigen could not detect same classes of immunoglobulin at different concentration as shown in Table (3). In my opinion ,the difference in the positivity of the sera may be due to the deletion of fragment 380 bp unfortunately there is no studies about this point,therefore I suggest it needs more study to evaluate. Or may be the factor of time during incubation who affect on the conditions in which the brucella are in.

The result in this study revealed that there was no significant statistical difference in antigen prepared which had equal quality, thus, the result obtained indicate the capability and the ability of preparing Rose Bengal antigens from BM. Encouraging using BM culture compared the performance of this BM with classical method showed similarity in addition its not preferable to leave cells in liquid broth for 24 hours and the simple principle of the biphasic growth technique appears to be applicable for biomass production regarding that GPIAMmedia incubation period ranges from 4 to 5 days. This method needs no special equipement, technologically simple and reagents are already available in most laboratories and requires little or no specialized training, simple to perform and reliable low cost alternative, eliminate the necessity of sub culture, minimize contamination and provides a level of safety. Our data highlight the support of using the BM method for the culturing of brucella species for preparation of antigen using in diagnosis of infected animals as alternative for classical Rose Bengal brucella antigen.

REFERENCES

1. Erdenebaatar J, Bayarsaikhan B, Yondondorj A, Watarai M, Shirahata T, Jargalsaikhan E, Kawamoto and K, Makino S. Epidemiological and serological survey of brucellosis in Mongolia by ELISA using sarcosine extracts. Microbiol Immunol 2004; 48: 571-577.
2. Macmillan A.P., Investigation of the performance of the rose Bengal plate test in the diagnosis of Brucella melitensis infection in sheep and goats. WAR/RMZ89 1997; 2: 57-60.
3. Corbel,M.J., Recent advances in Brucellosis. J. Med Microbiol 1997; 46: 101-103.
4. Refai M., Incidence and control of brucellosis in the near east region. Vet.Microbiol, 2002; 90: 81- 110.
5. Horn JK, Bacterial agents used for bioterrorism. Surg Infect, 2003; 4: 281-287.

6. Bernues A, Manrique E and Maza MT. Economic evaluation of bovine brucellosis and tuberculosis eradication programmes in a mountain area of spain.Preventive Veterinary Medicine 1997;30: 137-49.
7. Seleem MN, Boyle SM and Sriranganathan N., Brucellosis ; a re-emerging Zoonosis. vet Microbiol, 2010; 140: 392- 398.
8. World Health Organization.(WHO) Laboratory Safety Manual.3rd ed. Geneva, Switzerland, 2010.
9. Whatmore AM, Davison N, Cloeckaert A , Al Dahouk S, Zygmunt MS, Brew SD, Perret LL, Koylass MS, Vergnaud G and Quance C . Brucella Papionis sp. Nov., isolated from baboons (Papio spp). Int J Syst Evol Microbiol 2014; 64: 4120-4128.
10. Mantur BG, Amarnath SK and Shinde RS. Review of clinical and laboratory Features of human brucellosis. Indian J Med Microbiol, 2007; 25: 188-202.
11. Noviello S, Gallo R, Kelly M, Limberger RJ, DeAngelis K, Cain L, Wallace B, and Dumas N. Laboratory-acquired brucellosis. Emerg Infect Dis., 2004; 10: 1848-1850.
12. Young E.J. Human brucellosis. Rev Infect Dis., 1983; 5: 821-842.
13. Singh K., Laboratory – acquired infections. Clin. Infect. Dis., 2009; 49: 142-147.
14. Morgan W.J., The serological diagnosis of bovine brucellosis.Vet Rec., 1967; 80: 612-620.
15. Corbel M.J.: Comparison of Brucella abortus and Brucella melitensis Antigens for the rose bengl plate test on sera from cattle infected with B, abortus Biovar -5.Vet Rec., 1985; 117: 385-386
16. Sridhar Rao B.N. Bacterial culture media. www.microrao.com
17. May field J.E., JA Bantle, DR Ewald, VP Meader, Tabatabai. Detection of brucella cells and cell component in Nielson K. and JR Duncan {EDS}. Animal brucellosis. CRC press floride USA1996, 97-120.
18. Weinstein H.P. Current Blood culture methods and systems. Clinical concepts Technology and Interpretation of Results Clinical Infectous Diseasees, 1996; 23: 40.
19. Mangalogi, S., and SJJan A.J., Comparison of the three Blood culture techniques in the diagnosis of human brucellosis. Lab. Physicians, 2014; 6-1: 14-17.
20. Hestain, S., Avineri-Shapiro, S., and Aschner, M., The enzymatic production of levan. Biochem. J.(London), 1943; 37: 450-456.
21. Tyrrell, E.A.R.E. MacDonald, and P. Gerhard. T. Biphasic system for growing bacteria in concentrated culture. J.of Bacterial, 1958; 75: 1-4.
22. Ruiz- Castaneda, M.. A practical method for routine blod cultures Brucellosis. Broc. Soc. Exp. Biol. Med., 1947 64: 114-115.
23. Scott, E.G. A practical blood cultures procedure. Am.J.clinical Path., 1951; 21: 290-294.
24. Deva RAJ., Kali A, Pravin Charles MV and Seethe K.S. Modified Biphasic media for blood culture 2016 Asian J. of Pharm.& Clim Res., 9(3): 42-43.
25. P.Yagupsky J. Detection of Brucella in blood culture. Clinical Microbiol, 1999; 3437-3442.
26. OIE. Bovine Brucellosis Manual of diagnostic tests and vaccines for terrestrialanimals. World Organisation for Animal Health, 2016.
27. LeFleche, P., Jacques, M.Grayon, S.ALDahouk.P.Bouchon, F.Denoed, K.Nockler, H.Neubaue, L.A. Guilloteau and G.Vergnaud, Evaluation and selection of tandem repeat loci for a Brucella MLVA typing assay. B.M.C. Microbiol, 2006; 6.9.
28. Salmani AS, Siadat SD, Ahmadi H, Nejati M, Norouzian D, Tabaraie B, Abbasi M, Karbasian M, Mobarez, AM. and Shapouri, R,. Optimization of Brucella abortus S99 lipopolysaccharide extraction by phenol and butanol methods.Res. J. Biol. Sci., 2008; 3: 576-580.
29. Sambrook, J.; Fritisch, E. and Maniatis, T, Molecular cloning: A laboratory manual, secondedition. Cold spring Harbor Laboratory Press, 1989.
30. Kumar, S. Text book of Microbiology 2012 Jaypee Brothers Medical Publishas.
31. M.O.Salami, S.O.Alu, A.B. Opalekunde and O.A. Oladipo. Comparative Analysis between Monophasic and Biphasic Methods of Blood Culture. Journal of Biology, 2012; 2: 4.
32. Hornizky M and J Searson ,. The relation between the isolation of Brucella abortus and serological status of infected non-vaccinated cattle. Aust Vet J., 1986; 63: 172-174.
33. Alton.G.G: Jones. L.M: Angus. R.D.; and Verger, J.M Techniues for the brucellsis laboratory. Paris France:Institute National de La Recherche Agronomique, 1988; 17-136.
34. Al- Sulami A.: Hammadi S.S. and Al-ghazawi G.J. A step method for isolation and identification of Mycoplasma pneumoniae. Easter Mediterranean Health J., 2002; 1: 5-6.
35. Hendry, D.F.D., M.G. Corbel, R.A. Bell and G.A. Stack, Brucella antigen production and standardization Booklet 2499 1985. Minstry of Agriculture, Fisheries and Food, Lion House, Alwick, Northumberland: UK.
36. Phillips, M., B.L. Deyoe and P.C. Canning Protection of mice against Brucella abortus infection by inoculation with monoclonal antibodies recognizing Brucella O-A antigen. AM.J. Vet. Res., 1989; 50: 2158-2161.
37. Allardet-Servent A, G Bourg, M Ramuz, M Pages, M Bellis and G Roizes. DNA polymorphism in strains of the genus Brucella. J Bacteriol, 1988; 170: 4603-4607.
38. Mayer-Scholl A,A Draeger, C Gollner, HC Scholz and K Nockler, Advancement of a multiplex PCR for the differentiation of all currently described

Brucella species. J Microbiol Methods, 2010: 80:112-114.

39. Allan.G.S.; Chappel, R.J.; William, P. and Mcnaught, D.J. (Aquantitative comparison of the sensitivity of serological testsfor bovine brucellosis to different anmtibody clases. J.Hyg. Cam., 1967; 76: 287-298.