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Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) for Genotyping of Egyptian *Brucella* Isolates

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Abstract: In this comparative study, three *Brucella* vaccinal strains namely S19, RB51 and Rev-1 along with three *Brucella* reference strains designated as ETHER (*Brucella melitensis* biovar 3), 16M (*Brucella melitensis* biovar 1) and REO198 (*Brucella ovis*), in addition to 6 local isolates recovered from different animal species that proved to be *Brucella melitensis* biovar 3 biochemically and serologically. Multiplex PCR and MLVA were used to differentiate between the aforementioned *Brucella* strains. Multiplex PCR and MLVA using only three primer pairs to amplify three minisatellites loci in panel 1 were very successful and accurate in terms of characterization and typing of *Brucella* vaccines, reference and wild *Brucella* strains. Multiplex PCR and MLVA data were closely identical to those obtained by the traditional methods of identification. However, the molecular typing of the *Brucella* strains by multiplex PCR and MLVA had several advantages over the use of the conventional methods being very fast, precise, easier, more sensitive and economic and could be applied on minimal sample preparation. So, PCR and MLVA is a reliable tool in testing of the seed culture during the preparation of *Brucella* vaccines as well as in evaluating them in quality control laboratories, also powerful epidemiological tools for detection and diagnosis of brucellosis and helping in determining the situation of the disease.

Key words: *Brucella* · Brucellosis · Multiple Locus Variable Number Tandem Repeat Analysis · MLVA · VNTR and multiplex PCR

INTRODUCTION

Brucellosis, also known as “undulant fever”, “Mediterranean fever” or “Malta fever” is a zoonosis and the infection is almost invariably transmitted by direct or indirect contact with infected animals or their products. It affects people and animals of all age groups and of both sexes. Although there has been great progress in controlling the disease in many countries, there still remain regions where the infection persists in domestic animals and, consequently, transmission to the human population frequently occurs. It is an important human and animal’s disease in many parts of the world especially in the Mediterranean countries of Europe, north and east Africa, the Middle East, south and central Asia and Central and South America and yet it is often unrecognized and frequently goes unreported. There are only a few countries in the world that are officially free of the disease although cases still occur in people returning from endemic countries [1].

Brucellosis in animals was reported for the first time in Egypt at 1939. Serologically reactors cows recorded as 16.5 - 23.3%, whereas prevalence among buffaloes recorded as 7 - 10%.[2]. Intensive surveillance programs were initiated after the approval of the National Brucellosis Control Program in 1981, adopting the test and slaughter policy and vaccination of young female calves with the reduced dose of *B. abortus* S19 vaccine [3].

Nowadays, Prevalence of brucellosis in Egypt and Mediterranean countries is mainly due to infection of sheep and goats with *B. melitensis*. Despite bovine brucellosis caused by *B. abortus* has been nearly eradicated for several years, but currently cattle may potentially infect with *B. melitensis* [3]. A national

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program for control of brucellosis has been based upon vaccination of young animals with a living (naturally attenuated) vaccine (sheep and goats are vaccinated with *B. melitensis* Rev1, while cattle are vaccinated with *B. abortus* S19). Such control program include also surveillance, movement control within and outside herds, a test and slaughter policy for infected flocks and treatment of meat and milk products had been established [4-6].

Currently used methods for testing and identification of field brucella isolates or seed cultures used for production of brucella vaccines are based on conventional morphological, biochemical and serological methods besides growth on different stains and antibiotics containing media [7,8]. However, all these testes are time-consuming; require skillful technicians and some of the essential reagents that are usually not commercially available. To overcome most of these difficulties, this study aims to establish the use of a multiplex PCR designated as Bruce-ladder [9-10] and Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) assays [11-12] for molecular geno-typing of the field brucella isolates and seed cultures used for brucella vaccine production and as an epidemiological tracing approaches.

MATERIALS AND METHODS

***Brucella* Strains and Isolates:** Three lyophilized vaccinal strains [*B. abortus* biovar 1 (S19 and RB51) and *B. melitensis* biovar 1 (Rev-1)] were obtained from CZ veterinaria S.A., Pontevedra, Spain and three reference strains [*B. meltensis* biovar 1 (16M), *B. melitensis* biovar 3 (ETHER) and *B. ovis* (REO198)] were kindly supplied by Prof. Dr. JM Blasco, CITA Institute, Zargoza, Spain. Field isolate (no.1) which recovered from sheep (ewe) was previously identified traditionally and by using multiplex PCR in pervious study as *B. melitensis* biovar 3 [13] (Fig. 1). All other 5 local field isolates used in the study were of animal origin and were identified serologically and biochemically in this study as *B. melitensis* biovar 3. The details of all strains and isolates used in this study are presented in Table 1.

Table 1: *Brucella* strains studied (reference strains and field isolates)

Species	Biovar	Strain	Host
B. melitensis	1	Rev-1	Sheep
B. melitensis	1	16-M	Sheep
B. melitensis	3	ETHER	Sheep
B. abortus	1	S19	Cattle
B. abortus	1	RB51	Cattle
B. ovis		REO198	Sheep (ewe)
B. melitensis	3	Field isolate 1	Sheep
B. melitensis	3	Field isolate 2	Sheep
B. melitensis	3	Field isolate 3	Baffaloes
B. melitensis	3	Field isolate 4	Baffaloes
B. melitensis	3	Field isolate 5	Cattle
B. melitensis	3	Field isolate 6	Cattle

Multiplex PCR: In this study, a previously standardized multiplex PCR assay named Bruce-ladder was performed according to Garcia-Yoldi *et al.* [9] with limited modifications. In Bruce-ladder PCR five primers pairs (Table 2), designed on the strain-specific genetic differences and were used in multiplex PCR for molecular typing of different *Brucella* species. In this study, 12 multiplex PCR reaction mixtures each of 25 µl volume containing 1 µl of template DNA, 200 µM of each deoxynucleoside triphosphate (Fermentas), 2.5 units of DreamTaq$_{TM}$ Green DNA Polymerase (Fermentas), 5 µl of its amplification buffer and 20 pmole of each primer were used. The PCR amplification was carried out using GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, USA). The cycling conditions were 7 minutes at 95°C for initial denaturation, 25 cycles each of 30 seconds at 94°C for denaturation, 30 seconds at 64°C for primer annealing, 1 minute and 40 seconds at 72°C for extension of the amplicons and one cycle at 72°C for 7 minutes for final extension.

The PCR amplicons were analysed by running 10 µl of the PCR products in 1% agarose gel stained with ethidium bromide (0.5µg/ml). Thereafter, gels were photographed under UV illumination using gel documentation and analysis system supplied with starlight express MX516® 16-bit CCD camera and AAP-M5® software and amplification pattern of each *Brucella* species was determined according to molecular size of the amplified products using 100bp –Ladder [Stratagen Co., USA, Cat. No. 201115/ from 100 to 2000 bp fragments].

Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) Assay: A previously standardized MLVA was performed according to Le Flèche *et al.* [11]. In MLVA three primers pairs (Table 3) were used in 3 singleplex for molecular typing of different *Brucella* species. The different Tandem Repeats (TRs) are designated by using the nomenclature previously described [14]. For instance BRU211_63bp_257bp_2u (bruce11) is a TR at position 211 kb in the B. melitensis 16 M genome. Its common laboratory name (alias name)

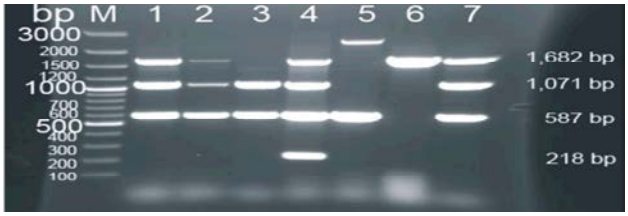

Fig. 1: Bruce-ladder multiplex PCR of vaccinal, reference and locally-isolated *Brucella* strains
M = 100 base pair (bp) DNA ladder (Fermentas). Lanes 1 to 7 are Bruce-ladder multiplex PCR amplicons of different *Brucella* species. Multiplex PCR of *B. melitensis* 16M and ETHER reference starins in lanes 1 and 2 respectively, *B. melitensis* Rev1 vaccinal strain (lane 4) and the *B. melitensis* (biovar 3) field strain isolated from an ewe in lane 7 produced three amplification products of 587 bp, 1071 bp and 1682 bp sizes with an additional 218 bp sized fragment in Rev1 strain only. Amplification products of *B. ovis* REO198 reference strain in lane 3 revealed two amplicons of 1071 and 587 bp sizes with the absence of the 1682 bp product. *B. abortus* RB51 vaccinal strain (lane 5) showed two amplicons of 587 bp and 2524 bp sizes and Lane 6 represents one amplicon of 1682 bp size for CZV-S.19 *B. abortus* vaccinal strain

Table 2: Primer sets for Bruce-ladder multiplex PCR

Primer	Sequence (5'–3')	Amplicon size (bp)	DNA targets	Source of genetic Difference
BMEI0998F	ATC-CTA-TTG-CCC-CGA-TAA-GG	1,682	Glycosyltransferase, gene *wboA*	S711 insertion in BMEI0998 in *B. abortus* RB51 and deletion of 15,079 bp in BMEI0993-BMEI1012 in *B. ovis*
BMEI0997R	GCT-TCG-CAT-TTT-CAC-TGT-AGC			
BMEII0843F	TTT-ACA-CAG-GCA-ATC-CAG-CA	1,071	Outer membrane protein, gene *omp31*	Deletion of 25,061 bp in BMEII826-BMEI0850 in *B. abortus*
BMEII0844R	GCG-TCC-AGT-TGT-TGT-TGA-TG			
BMEII0428F	GCC-GCT-ATT-ATG-TGG-ACT-GG	587	Erythritol catabolism, gene *eryC* (D-erythrulose-1-phosphate dehydrogenase)	Deletion of 702 bp in BMEII0427-BMEII0428 in *B. abortus* S19
BMEII0428R	AAT-GAC-TTC-ACG-GTC-GTT-CG			
BR0953F	GGA-ACA-CTA-CGC-CAC-CTT-GT	272	ABC transporter binding protein	Deletion of 2653 bp in BR0951 BR0955 in *B. melitensis* and *B. abortus*
BR0953R	GAT-GGA-GCA-AAC-GCT-GAA-G			
BMEI0752F	CAG-GCA-AAC-CCT-CAG-AAG-C	218	Ribosomal protein S12, gene *rpsL*	Point mutation in BMEI0752 in *B. melitensis* Rev.1
BMEI0752R	GAT-GTG-GTA-ACG-CAC-ACC-AA			

Table 3: List of tandem repeat loci investigated

VNTR	Alias name	Upper primer	Lower primer	B. mel.	B. abo.	No. of different alleles	Min-Max bp
BRU211_63bp_257bp_2u	Bruce11	CTGTTGATCTGACCTTGCAACC	CCAGACAACAACCTACGTCCTG	257	383	6	257-698
BRU424_125bp_539bp_4u	Bruce42	CATCGCCTCAACTATACCGTCA	ACCGCAAAATTTACGCATCG	539	289	5	164-789
BRU2066_40bp_273bp_3u	Bruce55	TCAGGCTGTTTCGTCATGTCTT	AATCTGGCGTTCGAGTTGTTCT	273	273	5	194-354

is Bruce11. It has a 63 bp motif and a total PCR product length of 257 bp in the Brucella melitensis 16 M strain when using the primer set indicated in (Table 3). This allele size corresponds to 2 units.

Brucella DNA was extracted using DNA extraction kits (BIO BASIC INC.). PCR amplification was performed in a total volume of 50 µl containing 1 µl of template DNA, 200 µM each of deoxynucleoside triphosphate (Fermentas), 2.5 units of DreamTaq$_{TM}$ Green DNA Polymerase (Fermentas), 5 µl of its amplification buffer and 20 pmole of each primer. Amplifications were performed in a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, USA). The cycling conditions were an initial denaturation step at 96°C for 5 minutes was

followed by 30 cycles of denaturation at 96°C for 30 sec., primer annealing at 60°C for 30 sec. and elongation at 70°C for 1 min. The final extension step was performed at 70°C for 5 min. Two to five microliters of the amplification product were loaded on a 3% standard agarose gel for analyzing tandem repeats with a unit length shorter than 10 bp and on a 2% standard agarose gel for all others [11] and run under a voltage of 8 V/cm until the bromophenol blue dye had reached the 20 cm position. Gels were stained with ethidium bromide, visualized under UV light, photographed and amplification pattern of each *Brucella* species was determined according to molecular size of the amplified products using [Gene rular100bp –Ladder, Fermentas Co., USA, Cat. No. SM0241/ from 100 to 3000 bp fragments].

RESULTS AND DISCUSSION

Identification of different members of *Brucella* species has been done with the traditional methods such as cultural, biochemical characterization and serological identification [7-8]. Recent methods are also introduced to the field of laboratory identification of brucella [15]. In the present study, 12 *Brucella* strains (6 local isolates, 3 vaccinal strains and 3 reference virulent strains) had been identified using traditional and recent methods. The local field isolates were recovered from different animal species clinically diagnosed positive for brucellosis. The vaccinal strains were S19 and RB51 which represent living vaccines against brucellosis originated from *B. abortus* while the third vaccinal strain was Rev-1 which represents living vaccine against brucellosis originated from *B. melitensis*. The three reference virulent strains were namely, 16M (*B. melitensis* biovar 1), ETHER (*B. melitensis* biovar 3) and *B. ovis* (REO198).

Traditional methods confirmed that S19 and RB51, each behaved as *B. abortus* biovar 1while Rev 1, 16M were behaved as *B. melitensis* biovar 1and ETHER strains was behaved as *B. melitensis* biovar 3 while The results of *B. ovis* REO198 was in accordance with behavior of *Brucella ovis* [7-8]. However, Egyptian field isolates were identified as *B. melitensis* biovar 3 (Table 1).

Molecular characterization trials have been carried out to differentiate the different *Brucella* species and biovars. DNA dependent methods have been recently nominated as tools for identification and differentiation of brucella isolates. While lots of methods were used for genomic characterization of brucellae, the restriction fragment length polymorphism (RFLP) and PCR was the most common.

In the present study, genomic DNA was extracted and a multiplex PCR assay was performed to identify as well as to differentiate brucella vaccinal strains from other *Brucella* species and biovars, including field isolates recovered from different animal species using previously designed five multiplex primer sets [9-10], which detect the unique genomic differences of *Brucella species* based on published whole genome sequences of *B. melitensis* [16], *B. suis* [17] and *B. abortus* [18], (Table 2).

A total of nine *B. melitensis* strains including the reference strains 16M and ETHER, the Rev-1 vaccinal strain and six field isolates recovered from different *Brucella*-infected animal species., where the infecting strains were biochemically identified as *B. melitensis* biovar 3, The PCR has amplified three fragments of 1682 bp, 1071 bp and 587 bp sizes for the Rev-1, 16M and the ETHER reference strains and the six field isolates. The *B. melitensis* Rev-1 vaccine strain was distinguished from other *B. melitensis* strains with a specific additional amplified fragment of 218 bp size produced by the BMEI0752 primer pair (Table 2). This BMEI0752 primer pair detects a unique point mutation in the rpsL gene, coding for the Ribosomal protein S12, of the vaccine strain *B. melitensis* Rev-1 and responsible for streptomycin resistance of Rev-1 [19]. The *B. ovis* REO198 strain produced two fragments of 587 bp and 1071 bp and distinguished by the absence of the 1682 bp fragment, which attributed to the failure of the BMEI0998 and BMEI0997 primers to anneal to target sequence because of a 15-kb deletion comprising omp25b and wboA-wboB genes in the *B. ovis* species [18, 20]. PCR of the S19 did not produce the 587 bp fragment, common to all *Brucella* species due to the deletion of 702 bp in the Erythroid catabolism gene eryC [21] leading to miss priming of the BMEII0428 primers pair. On the other hand, disruption of the *wboA* gene by an *IS711* element in *B. abortus* RB51 vaccinal strain led to the absence of the 1682 bp fragment characterizing the multiplex profile of RB51 strain. In addition, PCR of RB51 was characterized by a specific additional band of 2524 bp size consistent with the previous results reported by López–Goni *et al*. [10]. S19 and RB51 as all *B. abortus* species did not produce 1071 bp fragment encoding for omp31 (Fig. 2).

Multilocus Variable Number of Tandem Repeat (VNTR) Analysis (MLVA) was introduced in the last decade as a molecular typing tool for bacteria and has proven to be reliable and robust [22]. The technique was recently developed independently by three groups of investigators for typing *Brucella* isolates [11,12, 23]. One of advantages of this assay is that MLVA typing is

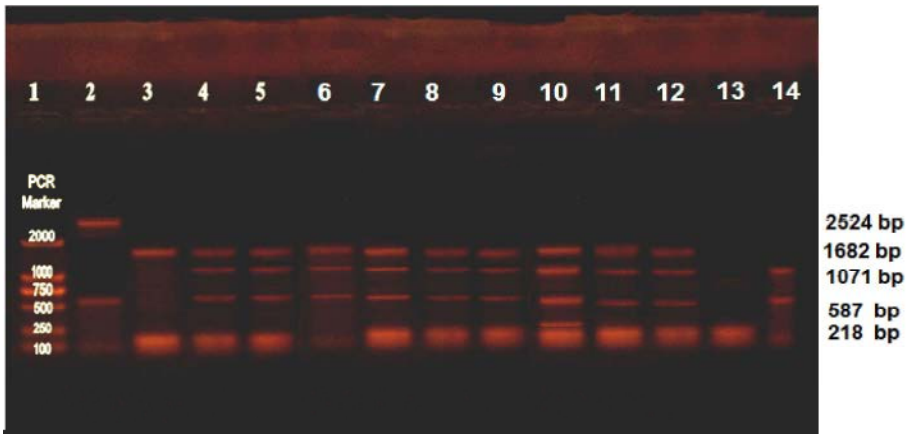

Fig. 2: Bruce-ladder multiplex PCR of vaccinal, reference and locally-isolated *Brucella* strains
Lane 1 = 100 base pair (bp) DNA ladder (Stratagen). Lanes 2 to 14 are Bruce-ladder multiplex PCR amplicons of different *Brucella* species. Multiplex PCR of *B. melitensis* 16M and ETHER reference starins in lanes 8 and 9 respectively, *B. melitensis* Rev1 vaccinal strain (lane 10) and *B. melitensis* (biovar 3) field isolates no. 1, 2, 3, 4, 5 and 6 respectively in lanes 4, 5, 6, 7, 11 and 12 produced three amplification products of 587 bp, 1071 bp and 1682 bp sizes with an additional 218 bp sized fragment in Rev1 strain only. Amplification products of *B. ovis* REO198 reference strain in lane 14 revealed two amplicons of 1071 and 587 bp sizes with the absence of the 1682 bp product. *B. abortus* RB51 vaccinal strain (lane 2) showed two amplicons of 587 bp and 2524 bp sizes and Lane 3 represents one amplicon of 1682 bp size for CZV-S.19 *B. abortus* vaccinal strain. Lane (13) is control negative

Fig. 3: Amplification patterns of MLVA (Bruce 11 singelplex) Lane 1 = 100 base pair (bp) DNA ladder (Fermentas). Lanes 2 to 14 are Bruce 11 MLVA singleplex of different *Brucella* species.
Lane 2:*B. melitensis* biovar 1 (Rev-1) Lane 3 *B. melitensis* biovar 3 (ETHER), Lane 4: *B. abortus* biovar 1 (RB51), Lane 5: *B. melitensis* biovar 1 (16M), Lane 6: field isolate 1, Lane 7: field isolate 2, Lane 8: *B. ovis* REO198, Lane 9: Lane *B. abortus* biovar 1 (S19), 10: field isolate 3, Lane 11: control –ve, Lane 12: field isolate 4, Lane 13: field isolate 5, Lane 14: field isolate 6.

only depends on the measurement of DNA amplicon sizes, so that a number of electrophoretic techniques can be used, ranging from manual, low-cost, agarose gels, to high-through-put capillary electrophoresis sequencing machines [11].

In this preliminary study, MLVA-3 assay which amplify 3 loci was selected as it can differentiated between different *Brucella* biovars on level of: *B. melitensis* biovar 1, 2 and 3 and *B. abortus* biovar (1, 2 and 3 as a group), 4 and (5,6 and 9 as a group), *B. ovis*, *B. suis* biovar 1, 2, 3,

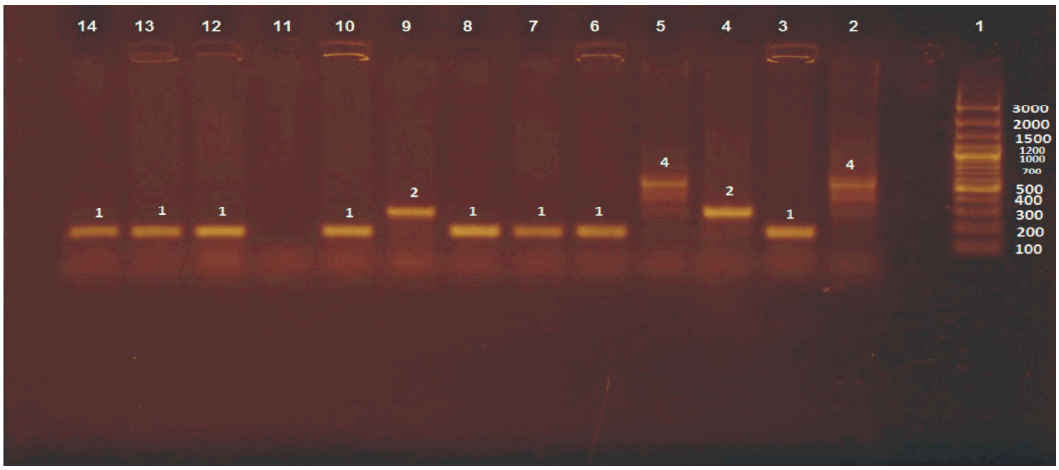

Fig. 4: Amplification patterns of MLVA (Bruce 42 singelplex) Lane 1 = 100 base pair (bp) DNA ladder (Fermentas). Lanes 2 to 14 are Bruce 42 MLVA singleplex of different *Brucella* species

Lane 2:*B. melitensis* biovar 1 (Rev-1) Lane 3 *B. melitensis* biovar 3 (ETHER), Lane 4: *B. abortus* biovar 1 (RB51), Lane 5: *B. melitensis* biovar 1 (16M), Lane 6: field isolate 1, Lane 7: field isolate 2, Lane 8: *B. ovis* REO198, Lane 9: Lane *B. abortus* biovar 1 (S19), 10: field isolate 3, Lane 11: control –ve, Lane 12: field isolate 4, Lane 13: field isolate 5, Lane 14: field isolate 6.

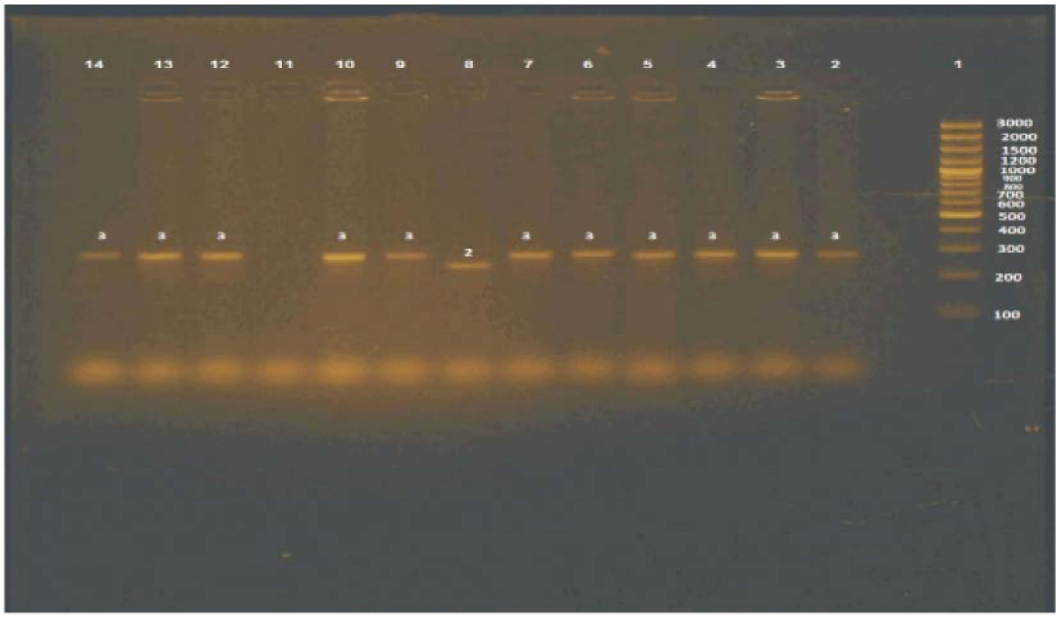

Fig. 5: Amplification patterns of MLVA (Bruce 55 singelplex) Lane 1 = 100 base pair (bp) DNA ladder (Fermentas). Lanes 2 to 14 are Bruce 55 MLVA singleplex of different *Brucella* species

Lane 2:*B. melitensis* biovar 1 (Rev-1) Lane 3 *B. melitensis* biovar 3 (ETHER), Lane 4: *B. abortus* biovar 1 (RB51), Lane 5: *B. melitensis* biovar 1 (16M), Lane 6: field isolate 1, Lane 7: field isolate 2, Lane 8: *B. ovis* REO198, Lane 9: Lane *B. abortus* biovar 1 (S19), 10: field isolate 3, Lane 11: control –ve, Lane 12: field isolate 4, Lane 13: field isolate 5, Lane 14: field isolate 6.

4 and 5, *B. canis*, *B. neotomae*, *B. pinnipediae* (marine spp.) and *B. cetaceae* biovar 1 and 2 (marine spp.) according to the results of [11]. This study included *Brucella* isolates recovered from different animal species.

The study included an insufficient number of isolates which enabled us to detect mutational events. Also, three minisatellite tandem repeats may assessed for mutational events which can differentiate *brucella* spp.

Table 4: MLVA-3 genotypes for 12 *Brucella* strains and isolates

Genotype	Bruce11	Bruce42	Bruce55
Rev-1	2	4	3
16-M	2	4	3
ETHER	3	1	3
S19	4	2	3
RB51	4	2	3
REO198	2	1	2
Field isolate 1	3	1	3
Field isolate 2	3	1	3
Field isolate 3	3	1	3
Field isolate 4	3	1	3
Field isolate 5	3	1	3
Field isolate 6	3	1	3

on biovar level but may be not enough to differentiate these biovars on genotype level especially these loci included 3 minisatellite markers in panel 1 that were useful for species identification, where a complementary group of microsatellite markers with higher discriminatory power located in panel 2 [24].

Anyhow this study is preliminary trial to establish the use of MLVA as a useful epidemiological tool for investigation of brucellosis in Egypt. The mutational activity (Polymorphism) of 3 VNTR loci across 6 field isolates of *Brucella* in addition to 6 references strains has been investigated and shown in Figs. 3- 5. The MLVA-3 typing assay allowed the 12 *Brucella* isolates to be grouped into 4 distinct genotypes and biovar. These 4 groups are *B. abortus* biovar 1 (RB51 and S19), *B. melitensis* biovar 3 (ETHER and the 6 field isolates), *B. melitensis* biovar 1 (16M and Rev-1) and *B. ovis* (RE198), as outlined in Table 4. The 3 loci were amplified with primers described by [11]. Bruce 11, 42 and 55 primer set amplified a 63, 125 and 40 bp basic repeat unit (repeat size) in the *Brucella* genome respectively. All 6 field isolates and ETHER strain had the same allele at the three loci with a single band of ~ 380, 160 and 310 bp produced with Bruce 11, 42 and 55 respectively while RB51 and S19 (vaccinal strains, *B. abortus* biovar 1) had the same allele at the three loci with a single band of ~ 450, 285 and 310 bp respectively 16M (Referance strain, *B. melitensis* biovar 1) and Rev-1 (Vaccinal strain, *B. melitensis* biovar 1) amplified fragments of 290, 540 and 310 bp size respectively. *B. ovis* REO198 produced 290, 160 and 270 respectively. Our results clearly show that *Brucella*, despite the high genetic homogeneity within the genus, is highly polymorphic at minisatellite levels.

In conclusion, Bruce-ladder PCR assay is recommended for testing the seed cultures commonly used in the production of living brucella vaccines (Rev-1, S19 and RB51 vaccines) and in evaluating them in quality control laboratories and also in identification and differentiation of *Brucella* isolates. Despite of the insufficient number of *Brucella* isolates, our results provide proof of the high discriminatory power of MLVA typing as this assay has the same advantages of multiplex PCR beside it can differentiate brucella isolates on biovar and sub-biovar level (genotyping). In the near future, it is tempting to speculate that international databases containing MLVA data of thousands of strains will be produced [25] and MLVA will become a routine assay for any new isolate and it is a useful tool for epidemiological tracing of brucellosis. Also, our findings confirmed that *B. melitensis* and in particular biovar 3, as the etiological agent most frequently isolated in Egypt.

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