Bulgarian Journal of Veterinary Medicine (2013), **16**, No 2, 112–122

PREVALENCE OF *LISTERIA* SPP. IN RAW MILK IN SYRIA

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Summary

Al-Mariri, A., A. Abou Younes & L. Ramadan, 2013. Prevalence of *Listeria* spp. in raw milk in Syria. *Bulg. J. Vet. Med.*, **16**, No 2, 112–122.

Testing for *Listeria* spp. was performed in milk samples collected from Syrian provinces, from August 2005 to December 2008. A total of 766 milk samples were collected from randomly selected dairy farms. Using conventional bacteriologic methods, 84 samples (10.96%) were positive for *Listeria* spp. The isolated *Listeria* spp. strains were catalase positive, oxidase negative, did not produce H_2S and were motile. The highest prevalence of *Listeria* spp. was found in raw bovine milk samples (16.2%), followed by sheep milk samples (12.4%), whereas *Listeria* spp. was not found in tested goat milk samples. *L. monocytogenes* was the most frequently isolated bacterium and was found in 41.6% of the raw milk samples. The remaining isolates were *L. innocua* (17.8%), *L. ivanovii* (14.2%), *L. welshimeri* (9.5%) and *L. gravi* (4.7%). PCR has confirmed our results using the specific *16S* rRNA gene and *16S-23S* spacer involved in the genotypic identification of *Listeria* sp. The results of this study indicated the potential risk of the infection with *Listeria* in people consuming raw and unpasteurised milk and dairy products.

Key words: API system, *Listeria*, PCR, raw milk

INTRODUCTION

Bacteria of the genus *Listeria* are widely distributed in the environment and they frequently contaminate food. *Listeria* are aerobic non-sporulating rods, microaerophilic and facultatively anaerobic and can be cultured over a wide temperature range (Johnson *et al.,* 2004). *Listeria* have been isolated from a variety of materials including soil, water, plants, faeces, decaying vegetables, meat, seafood, dairy products, and from asymptomatic human and animal carriers (Hain *et al.,* 2007). For many years, the genus *Listeria* contained only one species, *L. monocytogenes*. There are currently six recognised species including *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii* and *L. grayi* (McLauchlin, 1997; Hain *et al.,* 2006a). All listerial genomes sequenced to date are circular chromosomes with sizes that vary between 2.7 and 3.0 Mb. In comparison with the genome sizes of *L. monocytogenes*, *L. innocua*, *L. seeligeri*, and *L. ivanovii* (2.7–3.0 Mb), *L. welshimeri* has one of the smallest genomes of the genus *Listeria* (Hain *et al.*, 2006b). Two of the species are pathogenic, causing the disease listeriosis. Although there are six distinct species, they all have similar genetic homology which helps explain their similar phenotypic traits. Haemolysis as well as acid production are key characteristics in distinguishing the species (Bille *et al.,* 1999).

At present, only strains of *L. monocytogenes* are pathogenic to humans and

animals, while *L. ivanovii* are only pathogenic to animals, particularly ruminants such as sheep and cattle (Vazquez-Boland *et al*., 2001). *L. monocytogenes* is known to be secreted in milk by both infected and healthy animals (Wagner *et al.*, 2000), and contamination of milk may also be due to environmental contamination from farm environment and faecal material due to poor hygiene practice (Hassan *et al*., 2001). The food products most frequently associated with listeriosis are soft cheeses, particularly those made from unpasteurised milk, and ready-to-eat meat-containing food products (Kaclikova *et al.*, 2001).

Human listeriosis is a food-borne disease, and it has been estimated that 99% of all human listeriosis cases are caused by the consumption of contaminated food products (Mead *et al*., 1999). Although listeriosis is not common in humans, it is a clinically significant disease because of its high mortality and severity (Atil *et al*., 2011). The mortality rate is considerably higher than the more common infections from other food-borne pathogens such as *Escherichia coli* O157:H7, *Campylobacter* spp. and *Salmonella* spp. (Mead *et al.*, 1999). However, because the threats to public health posed by contamination of foods by each of these *Listeria* species are not similar, it is very important that they are rapidly and reliably detected and identified. Standard identification of *Listeria* from samples contaminated with multiple species relies on selective enrichments and subsequent biochemical analysis and can be difficult, labourious, and time-consuming; in many instances requiring 5 to 10 days (Manzano *et al.*, 1997; Holko *et al.,* 2002; Almeida & Almeida, 2000). The identification of *Listeria* species has long been hampered by the small number of tests allowing the differentiation between these closely related species. The reliability of PCR detection methods depend, in part, on the purity of the target template and the presence of sufficient numbers of target molecules. With such complex matrices as foods, steps must be taken to limit the effects of any potentially inhibitory compounds present that may limit PCR amplification of the intended target (Amagliani *et al*., 2007). To avoid these drawbacks, faster PCR identification procedures targeting the *16S* rRNA gene are used. On the other hand, the *23S* rRNA molecule includes a larger number of phytogenetically meaningful sites, and thus offers a supplemental target for achieving a higher degree of differentiation. Further, the rRNA intergenic spacer (IGS) regions are additional tool for bacterial determination (Bubert *et al.,* 1999; Manzano *et al.,* 2000).

The objective of this study is to determine the level of contamination by isolating and identifying the *Listeria* species in 766 raw milk samples from Syria.

MATERIALS AND METHODS

Milk samples

A total of 766 samples of raw milk were collected from several Syrian provinces (Damasccus, Rural Area, Daraa, Al-Sweida, Quneitra, Hama, Al-Hasakeh, Deir Azzor, Tartous, Lattakia, Aleppo, Idleb) between August 2005 and December 2008. Upon collection of the raw milk samples into sterile tubes and transportation to the laboratory the isolation studies commenced on the same day.

Isolation of Listeria species

For each sample, 25 mL of milk were added to 225 mL *Listeria* Selective Enrichment Broth (MERCK, Germany). The mixture was then homogenised in the medium by shaking for 2–3 min. Inoculated

Table 1. Nucleotide sequences of used primers sets

Gene Target	Primer sequence (5'–3')	Product size (bp)	*Listeria* specificity
16SrRNA	For1: GATAAGAGTAACTGCTTGTCCCTT Rev1: CCCTAACACTTAGCACTCATCGT	400	*Listeria* genus
16S-23SrRNA spacer	For2: TCATTCGCTCACACCGTAAA Rev2: TCATTAGCACCTGGTGTCAGA	380	*L. welshimeri*
16S-23SrRNA spacer	For3: CTTAAAAGACCGCCTGCGCG Rev3: GATAAGAGTAACTGCTTGTCCCTT	200	*L. gravi*
16S-23SrRNA spacer	For4: CTTAAAAGACCGCCTGCGCG Rev4: GGGAATCTTCCGCAATGGAC	200	*L. ivanovii*
16S-23SrRNA spacer	For5: AATTCCCACAGGACACAACC Rev5: CGGGAATGCAATTTTTCACTA	380	*L. innocua*
16S-23SrRNA spacer	For6: CCGTGCGCCCTTTCTAACTT Rev6: TTTGTTCAGTTTTGAGAGGT	400	*L. monocytogenes*

media were then incubated at 30 ºC for 24–48 h. After that, the solid culture was planted on *Listeria* identification Agar Base (PALCAM) and *Listeria* Oxford Medium Base, and incubated for 48 h at 37 ºC under aerobic conditions. Afterwards, planted plates were evaluated using Henry method of oblique lighting (Iordache & Tofan, 2008). Each colony 1–3 mm in diameter with black haloes was considered typical. Typical *Listeria* colonies were separated, planted in Trypticase soy agar (MERCK, Germany) and incubated for 24 h at 37 °C.

The *Listeria* were identified by using the following tests: Gram staining, catalase production, oxidase activity, H_2S production using Triple Suger Iron Agar (MERCK, Germany), and mobility test in SIM media at 25 °C and 35 °C (Doyle *et al.,* 1997). The colonies isolated as *Listeria* were then subjected to β-haemolysis and CAMP test in a 7% sheep blood agar for species identification (Aygun & Pehlivanlar, 2006).

Identification of Listeria

Isolates were biochemically confirmed as *Listeria* spp. The biochemical characteristics of the examined samples were determined by using the API *Listeria* system (Biomérieux, Marcy l'Etoile, France) to identify the *Listeria* strains, based on the presence or the absence of arylamidase (DIM test), hydrolysis of esculin, presence of ox-mannosidase, and acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-tagatose. All isolates were cultured overnight in Trypticase soy broths (MERCK, Germany) then added individually to the substrate in wells of the API strips. The strips were incubated at 37 °C and then monitored for changes in the color of medium after 24 h. Isolates discrimination was based on the principle of a pattern matching manual as described by the manufacturer. Occasionally, acid production from some carbohydrate fermentations was difficult to interpret; therefore, the tests were repeated with these strains to facilitate the reading of the indicator colour change.

DNA extraction and PCR conditions

Briefly, 1 mL of pure culture of *Listeria* was centrifuged at 13,000×*g* for 5 min at room temperature. The DNA was then extracted using a genomic DNA purification kit (Fermentas, GmbH, Germany) according to the manufacturer's protocol. Oligonucleotide primers for the PCR assay were selected based on the published nucleotide sequence of the 16s RNA and 16s/23s rRNA IGS regions amplification (Table 1). A 25 µL aliquot of PCR buffer contained 22 µL of PCR supermix (1.5 µL PCR reaction buffer, 3 µL $MgCl_2$ (25 mM), 0.5 µL of each dNTP, 1 µL of each primer, 2 µL bacterial genomic DNA solution and 1 µL Taq DNA polymerase in 16 µL of double-distilled H_2O). Three µL of each supernatant were added to the PCR mix. Thermocycling conditions included an initial hold of 2 min at 94 °C, then a denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s and a 30 s extension at 72 °C for a total of 30 cycles. A final hold at 4 °C followed a final extension at 72 °C for 10 min. Amplification reactions were carried out using a GeneAmp® PCR System 9700 (Applied Biosystems). The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with 1% solution of ethidium bromide, and examined under UV illumination.

Statistical analysis

Data were transferred to a Microsoft Excel spreadsheet for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), a chi-square test and Fisher's exact two-tailed test analysis were performed and differences were considered significant at values of P<0.05.

RESULTS

Isolation of Listeria bacteria

Listeria spp. was isolated from 84 (10.96%) of samples, mainly in Damascus and its rural area (57.6%), as well as Aleppo and Idleb (51.3%) provinces. In

Table 2. Samples of milk collected from different Syrian provinces

Province	Cow milk		Goat milk		Sheep milk	
	n	positive n (%)	n	positive n (%)	n	positive n (%)
Damasccus and rural Area	42	14 (33.30%)	–	–	37	9 (24.30%)
Daraa and Al-Sweida	45	–	47	–	35	–
Quneitra	55	8 (14.50%)	51	–	32	4 (12.50%)
Hama	58	6 (10.30%)	55	–	39	5 (12.80%)
Al-Hasakeh and Deir Azzor	65	4 (6.15%)	43	–	31	3 (9.70%)
Tartous and Lattakia	43	7 (16.30%)	–	–	24	5 (20.80%)
Aleppo and Idleb	37	17 (45.90%)	–	–	27	2 (7.40%)
Total	345	56 (16.23%)	196	–	225	28 (12.44%)

our study condition, we couldn't detect *Listeria* in goat milk samples. Table 2 demonstrates the regional distribution of *Listeria* isolated from cow and sheep milk.

API assay

The DIM results for all strains tested were unequivocal. Three biochemical characteristics were positive in almost all isolates (>95%) including hydrolysis of esculin and acid production from D-arabitol and a-methyl-D-glucoside (except for *L. grayi*).

Table 3 shows that the most commonly isolated *Listeria* species was *L. monocytogenes* (41.6%). The prevalences of remaining isolates were *L. ivanovii* (14.3%), *L. innocua* (17.8%), *L. welshimeri* (9.5%), *L. gravi* (4.7%), and *Listeria* spp. (11.9%).

Our results showed that among the *Listeria* isolates, which are grown on sheep blood agar, 35 *L. monocytogenes* isolates and 12 *L. ivanovii* isolates were haemolytic.

PCR results

Fig. 1 demonstrates the length of *16sRNA* gene when using the For1 and Rev1

Table 3. *Listeria* spp. isolated from cow and sheep milk samples

Strains	n (%)	Cow milk		Sheep milk	
		n	(%)	n	(%)
L. monocytogenes	35 (41%)	23	65.71	12	34.29
L. innocua	15 (18%)	11	73.34	4	26.66
L. ivanovi	12 (14%)	7	58.33	5	41.67
L. gravi	4 (5%)	3	75.00	1	25.00
L.welshimeri	8 (10%)	5	62.50	3	37.50
Listeria spp.	10 (12%)	7	70.00	3	30.00
Total	84 (100%)	56	66.67	28	33.33

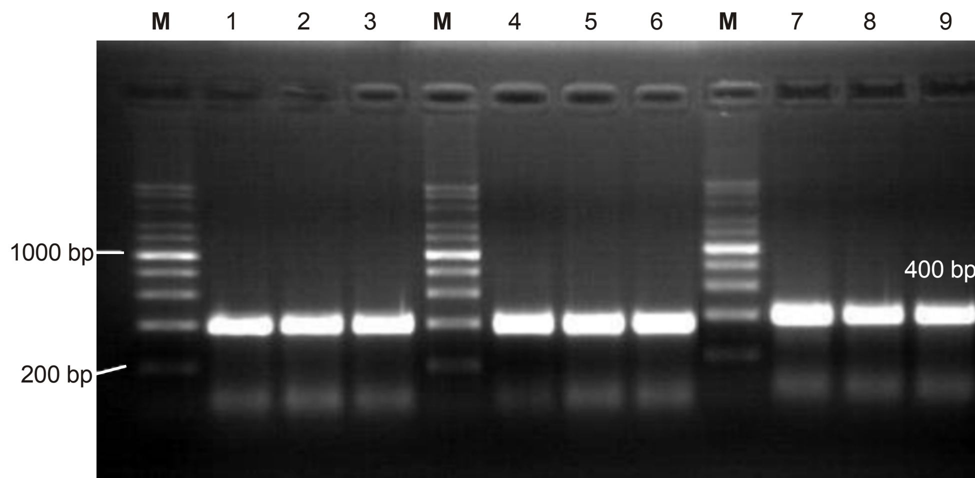

Fig. 1. The length of *16sRNA* gene using specific primers: Electrophoresis on 1.5% agarose gel showing the PCR products. Lane 1 corresponds to *L. monocytogenes* ATCC 98 positive control (400 bp), whereas lanes 2, 3, 4, 5, 6, 7, 8 and 9 correspond to our isolates.

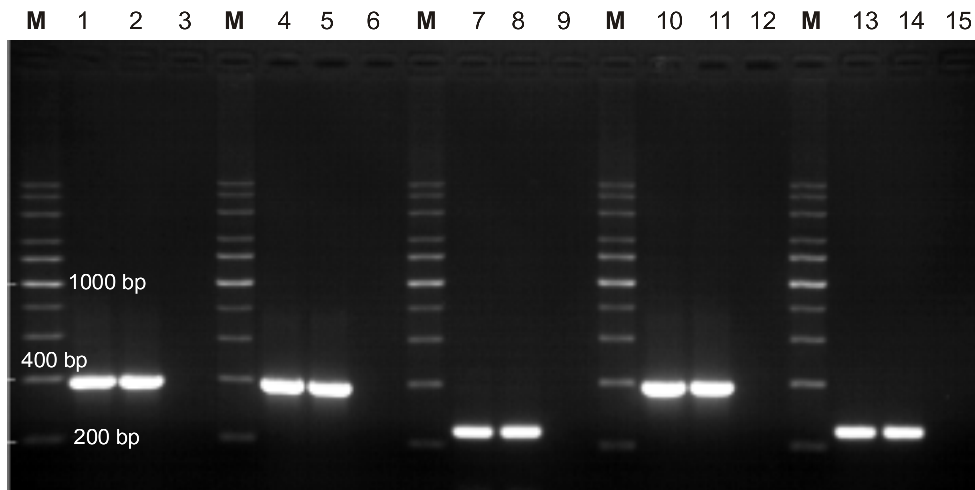

Fig. 2. Results of electrophoresis on 1.5% agarose gel. 100-bp DNA ladder (lane M); *L. monocytogenes* (lanes 1 and 2); *L. innocua* (lanes 4 and 5); *L. ivanovii* (lanes 7 and 8); *L. welshimeri* (lanes 10 and 11); *L. gravi* (lanes 13 and 14). Lanes 3, 6, 9, 12 and 15 correspond to negative controls.

specific primers. Bands 2, 3, 4, 5, 6, 7, 8 and 9 corresponding to our isolates have shown the same length as *L. monocytogenes* ATCC 98 positive control (400 bp). Thus we may suggest that our isolates were from *Listeria* spp. genus.

Fig. 2 summarises the PCR results of the isolates. The *L. monocytogenes* specific 400 bp amplification product was visualised in the contaminated raw milk samples (lane 1). In addition, the 380 bp band corresponding to *L. innocua*, and the 200 bp band corresponding to *L. ivanovii* were shown in lane 4 and lane 7, respectively. However, the For2 and Rev2 primers (380 bp) were specific and corresponding to *L. welshimeri* (lane 10); and the For3 and Rev3 primers (200 bp) were specific for *L. gravi* (lane 13). *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. gravi* (lanes 2, 5, 8, 11, 14) were used as positive controls.

DISCUSSION

The true presence of listeriosis in animals or food is not known in Syria since there have not been comprehensive studies conducted on *Listeria* and listeriosis in this country. Most cattle and sheep farms in Syria do not have adequate hygiene precautions and these animals live in a natural environment together with people. Therefore, we aimed at the determination of the prevalence of *Listeria* spp. in Syrian cow, sheep and goat milk.

In the present study, 84 of the 766 raw milk samples (10.96%) were positive for *Listeria* spp. Therefore, the contamination source of *Listeria* spp. in raw milk in this case is likely due to mistreatment of milk, and to lack of cooling process after milking, especially in small farms (Sagun *et al.*, 2001). The source of *Listeria* spp. in raw milk has been reported to be faecal (Aygun & Pehlivanlar, 2006) and environmentally contaminated during milking, storage and transport, infected cows in dairy farms and poor silage quality (Bemrah *et al.*, 1998). The reason behind investigating *Listeria* spp. in this study is its importance to the dairy products sector. Another reason of concern is that some farms are producing their own

cheeses directly from untreated milk. These results show the need for improved control and epidemiologic strategies to prevent the transmission of *Listeria* spp. to consumers. Furthermore, our results are consistent with previous studies where the incidence of contaminated milk samples varies among countries, being 1.2% in Denmark (Jensen *et al.,* 1996), 3.62% (of 774 samples) in Spain (Gaya *et al.,* 1998). In 2006 Aygun & Pehlivanlar investigated the presence of *Listeria* spp. in a total of 157 raw milk samples sold in Antakya (Antioch), and it was found to be 2.12%. Ahrabi *et al*., in a study conducted in 1998 concluded that the total incidence of *Listeria* in milk samples was 10% from different regions of Anatolia. It was 4.5% in Korea between 1993 and 1997, whereas the prevalence of *L. monocytogenes* reported by Bhilegaonkar *et al.* (1997) in Northern India was 8.1% of 86 bovine raw milk samples tested. In our study, 10.96% of raw milk samples were positive of *Listeria* spp., in which *L. monocytogenes* was detected in 41.6% of samples, *L. innocua* in 17.8% of samples, *L. ivanovii* in 14.2% of samples, *L. welshimeri* in 9.5% of samples and *L. gravi* in 4.7% of samples. In a study on dairy products, Pak *et al.* (2002) found the incidence of *L. monocytogenes* to be 4.9%. Carlos *et al.* (2001) in Mexico city, studied the incidence of *Listeria* spp. in 1300 raw milk samples, and found that 13% were positive for *L. monocytogenes*, 6% for *L. ivanovii*, 4% for *L. seeligeri* and 1% for *L. innocua*. However Gaya *et al.* (1996) found that the incidence of *L. monocytogenes* was 2.56%, *L. innocua* 1.73% and *L. ivanovii* – 0.21%. A study on milk in England by Greenwood *et al.* (1991) established an incidence of *L. monocytogenes* of 8.2%. In this study, the findings show that the incidence of *L. monocytogenes* in Syria was 41.6%. It is a rather high value as compared to values obtained from around the world. Also, the results demonstrated that the incidence of *L. ivanovii* in our country was 14.3% too.

There is some concern about the presence of *L. ivanovii* in milk for certain strains of this species, which have been shown to be pathogenic in hens, sheep and cattle (Allerberger, 2003). In this research, we couldn't detect *Listeria* spp. in goat milk contrary to the study conducted about goat milk in Italy when ninety-six samples of bulk untreated goat milk were subjected to exhaustive micro-biological analyses for the presence of *Listeria* spp. and 2.1% of samples were found positive for *Listeria* species (*L. innocua* or *L. ivanovii*) (Soncini & Valnegri, 2005). All these investigations have identified raw milk as a source of *Listeria* spp., but environmental and faecal contamination during the transportation of milk and its storage have also been reported (Bemrah *et al.,* 1998; Frece *et al*., 2010).

Statistical analysis on the variation in *Listeria* spp. showed no significant differences between the isolated *Listeria* spp. and the covered area in this study whatever the source of milk was, whether from cow or sheep. Additional statistical study was applied on isolated *Listeria* spp. as related to the seasons between 2005 and 2008. However, the results showed no significant difference in this regard. Moreover, it is concluded that there is no relationship between raw cow or sheep milk pollution and *Listeria* spp. among sampling regions during the study period. Nevertheless, these results disagree with Gaya *et al.,* (1996) and Waak *et al.,* (2002), who reported that the raw milk pollution with *Listeria* is related to the season. This variation may stem from the animal feeding quality of five months on fresh green feeds and on processed dry hay prepared from the

vegetation of the farm itself for the rest of the year.

Recent epidemiological investigations of foodborne outbreaks of human listeriosis and the subsequent regular control of various foods have clearly established an acute need for effective methodology for a rapid, simple, and reliable system of identification of *Listeria* isolates that is easily applicable to large sample numbers. Because of the time-consuming nature of traditional identification procedures, some commercial identification systems such as API, have been evaluated in this view (Bille *et al.,* 1992). Spontaneous haemolysis, a major characteristic for *Listeria* species identification, may, in some cases (and especially for environmental and food isolates), be difficult to read on blood agar plates when differentiating *L. monocytogenes* from *L. innocua*. To circumvent this important problem, more sensitive methods like the use of microtiter plates, a CAMP test could be used. Nevertheless, even with these tests, some true nonhaemolytic *L. monocytogenes* isolates might be misidentified as *L. innocua* on the basis of their phenotypical behavior. To date, only genomic methods can firmly identify such isolates, and differentiate between the closely related species, such as technology of polymerase chain reaction PCR, and much discussion has taken place about the advantages of PCR techniques, their specificity, reliability and rapidity in the detection of food borne pathogens, and identification of *Listeria* isolates. Indeed, sequence analysis of the IGS regions of 16S–23S rRNA operons has been extremely rewarding in respect to the genotypic identification of bacteria species including *Listeria.* Interestingly, *Listeria* 16S–23S IGS regions are of two different sizes: a smaller one of about 340 bp and a larger one of 550–590 bp. The small rRNA IGS regions of *L. innocua, L. ivanovii, L. seeligeri, L. welshimeri,* and *L. grayi* show 83–99% homology to that of *L. monocytogenes*; the large rRNA IGS region of *L. monocytogenes* demonstrates a 81–96% similarity to those of non-*monocytogenes Listeria* species (Bubert *et al.,* 1999; Graham *et al.,* 1996; Manzano *et al.,* 2000; Winters *et al.,* 1999). PCR, as well as the API, showed a higher sensitivity than other conventional methods in the detection of *Listeria* species, which are found in a very small number in food material. The results were in good agreement for the 766 raw milk samples tested for *Listeria* spp., by both the API and PCR; all 84 positive and 682 negative samples were detected by both methods.

Finally and based on the above results, it may be concluded that we need more research to control programmes for interactions among feeds, animals and *Listeria*. More detailed genetic research should also be performed to compare *Listeria* spp. isolates originating from different sources such as animals, feeds, humans and the environment.

ACKNOWLEDGEMENTS

The authors would like to thank the Director General of the AECS and the Head of the Molecular Biology and Biotechnology Department for their support.

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Paper received 04.02.2013; accepted for publication 18.03.2013

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