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Molecular Taxonomy Among *Mentha spicata*, *Mentha longifolia* and *Ziziphora tenuior* Populations using the RAPD Technique

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Abstract

The Random Amplified Polymorphic DNA (RAPD) technique was used to study the molecular taxonomy and genetic relationship between two *Mentha* species namely, *Mentha spicata* and *Mentha longifolia*, and *Ziziphora tenuior*. Sixteen RAPD primers showing polymorphic bands were used for the construction of the dendrogram and a similarity matrix. A total of 2001 bands were obtained; 419 of them were polymorphic. Similarity values among the studied samples ranged from 0.68 to 0.03. High similarity values were obtained between two samples of *Mentha spicata* (0.68) collected from local markets and between three samples of *Mentha longifolia* (0.64) as well. RAPD analysis confirmed that *Mentha* species are genetically different from *Ziziphora tenuior* and a genetic variation was found between and within the species tested for this study. The cluster analysis clearly differentiated *Mentha spicata and Mentha longifolia* from *Ziziphora tenuior*. Molecular analysis with RAPD markers stressed their ability for differentiation between families, genus, and species of living organisms particularly *Mentha* species and *Ziziphora tenuior*.

Keywords: *Mentha, Ziziphora*, RAPD, Taxonomy, Jordan.

1. Introduction

Jordan has a rich flora of medicinal plants with diverse biological properties. *Mentha* L. species are one of the most important medicinal and aromatic plant species used in Jordan and worldwide. These are sources of essential oils that are widely used in food, flavour, cosmetic and for pharmaceutical purposes. *Mentha* (M.), is the most important genus of aromatic perennial herbs belonging to the Labiatae (Lamiaceae) family and distributed mostly in temperate and sub-temperate regions of the world. It contains a number of taxa with high economic essential oils and within this section *Mentha*, five basic Eurasian and African species (*M. arvensis* L., *M. aquatica* L., *M. spicata* L., *M. longifolia* (L.) Huds., and *M. suaveolens* Ehrh.) have been identified, with eleven naturally occurring named hybrids (Lawrence, 2007; Bhat *et al*., 2002). The species of section *Mentha* typically have chromosome number 2n=2x=12, but the other species vary widely, with *M. spicata* L. and *M. longifolia* have 2n=2x=48 and 2n=2x=24, respectively (Lawrence, 2007; Murray, 1960).

The spearmint, *M. spicata,* is a hybrid of *M. longifolia* and *M. rotundifolia*, morphological, cytological and biochemical data have shown that the tetraploid species of *M. spicata* (2n=48; Lawrence, 2007) originated by chromosomal doubling of hybrids between the two closely related and inter-fertile diploids, *M. longifolia* and *M. suaveolens* (Harley and Brighten, 1977). The ketone constituent of the oil is important in three ways; with one of them is to give the oil and herbage its characteristic odor (Murray, 1960). Oil from an individual of the polymorphic species *M. spicata* may have any (but only one) of the three ketone groups (Murray, 1960). The chemical constituents in the oil of *M. spicata* were 58% carvone, 8% limonene, 10% dipentene, 7% dihydrocarveol, and it can be used in foods, beverages, tooth paste mouth wash, soaps, detergents and perfumes and medicinally as stimulant carminative, anti-spasmodic and in bronchitis and fever (Bhat *et al.,* 2002). The essential oil of *M. spicata* showed good activity against larvae of fourth instar of Anophel stephensi (Hadjiakhoond *et al.*, 2000) also it has radical scavenging activities (Souri *et al.,* 2008). At the level of folk medicine, the leave decoction of *M. spicata* can be taken twice a day for a week to cure throat infection and indigestion (Mahato and Chaudhary, 2005). Mint is usually taken after a meal for its ability to reduce indigestion and colonic spasms by reducing gastrocholic reflux (Bhat *et al.,* 2002). In Egypt, *M spicata* is cultivated for its volatile oils; it is also used in food flavoring (Bader *et al.*, 2003), as a culinary herb, and in toothpaste and chewing gum industry (Naghibi *et al.,* 2005).

The very musty odor of *M. longifolia* (L.) Huds. (2n=2x= 24) is that of pure piperitone oxide, its principal ketone. This species has smaller amounts of the related

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ketone, piperitenone oxide (Murray, 1960). *M. longifolia* has 56% piperitone oxide, 20% pipertenone, disophenol, disohenolene (in traces) and is mainly used for treatment of nausea, gastralgia, neuralgia rheumatism, bladder stone, gall stone, rheumatism, jaundice, diarrhoea, toothache, stomachache, anti-infection, dyspnea, flatulence, gastrodynia, dyspepsia, sedative, stomach tonic, insect repellent and headache as well as its being used as a vegetable in most parts of Iran, especially in the Northern region (Bhat *et al*., 2002; Naghibi *et al.,* 2005). The essential oil of *M. longifolia* has important compounds (menthol, menthone, pulegone,) having interesting antimicrobial activities, after 24 h of bacteria treatment with *M. longifolia* essential oil, they noted a big damage in *S. typhimurium* and *E. coli* (rod bacteria), whereas damage is less important in coccoid bacteria (*M. luteus* and *S. aureus*) (Hafedh *et al.,* 2010).

The genus *Ziziphora* (Z.) belongs to the family Labiatae and consists of four species (*Z. clinopodioides* Lam., *Z. captitata* L., *Z. persica* Bunge. and *Z. tenuior* L.) that are widespread all over Iran. *Z. clinopodioides*, with the common Persian name "kakuti-e kuhi" is an endemic species and grows wild in Iran, Afghanistan, Iraq, and Talish (Verdian-Rivi, 2008). *Z. tenuior* is distributed in a defined area particularly at southern part of Jordan. It has an attractive odor and the local communities use it to make tea. *Z. tenuior* is a common teapot herb and used for treatment of fever, dysentery, coughing, diarrhea, painful menstruation, bladder stone, abortifacient and stomach tonic (Naghibi *et al.,* 2005).

In Jordan, Al-Quran (2005) reported that the largest genera was *Mentha* including: *M. aquatica, M. graveolens* L., *M. longifolia, M. piperita* L., *M. pulegium* L. and *M. spicata.* In the past, seed protein analysis and morphology were used for taxonomy and evolutionary studies between and within species and subspecies levels. Šarić-Kundalić *et al.*,(2009) conducted a taxonomic study on the anatomical, morphological and photochemical differentiation of the genus *Mentha* L. (Lamiaceae) in Bosnia & Hercegovina and Slovakia. Nowadays, molecular markers have been used to define the species relatives and their taxonomy. Among them, RAPD and AFLP have the utility of being used as a means of studying taxonomy and genetic diversity among different *Mentha* species (Gobert, *et al.,* 2002; Khanuja, *et al.,* 2009 and Shasany, *et al.,* 2005). This study aims at studying the molecular taxonomy and the genetic relationships among two species of *Mentha* namely M. *spicata* and *M. longifolia* and *Z. tenuior* , using RAPD molecular analysis.

2. Materials and Methods

2.1. Plant material

This study includes a total of 30 samples of *Mentha* species composed of 10 samples of *M. spicata* collected from local markets, 10 samples of a wild *Z. tenuior* collected from Al-Shoubak district, and 10 samples of a wild *M. longifolia* collected from the flow of the Hussban stream in Jordan during 2009/2010 to be used for molecular taxonomy based on RAPD analysis. DNA analysis was conducted at the National Center for Agricultural Research and Extension (NCARE).

2.2. DNA isolation

Total cellular DNA was extracted following the procedure as described by Doyle and Doyle (1987), with minor modifications. Approximately 20 mg of fresh leaves of *Mentha* samples were ground in liquid nitrogen and mixed with 600 µl of freshly preheated 2x CTAB solution with 0.8g PVPP in 2ml tubes then placed at 65ºC for 30 min. The mixture was added to 600 µl of chlorophorm/ isoamyl alcohol (24:1), vortexed for few seconds, and then centrifuged at 13,000g for 10 min. The supernatant was placed in 2ml tubes with 600 ml isopropanol, and then shaken until the threads of DNA appeared, then centrifuged for 10 min at 13000g. The solution was poured from the tubes, and the pellet was left to dry. 600 µl of cooled 70% ethanol was added to the pellet and was placed in the refrigerator (–20ºC) overnight. Next day, ethanol was poured from the tubes, the pellet was allowed to dry and 150µl of TE was added and the whole mixture was placed at 65ºC for 30min. Four micolitrers of RNAase (10mg/ml) were added per tube and incubated for 60 min at 37ºC. DNA quantity was measured using a S2100 UV/VIS DIODE-Array-Spectrophotometer, machine Version 1.7.

2.3. PCR amplification

The PCR reaction was performed as described by Williams *et al*. (1990) with 10-mer ologonucleotides synthesized by Operon technologies (Almeda, Calif.). The final PCR volume of 25 µl contained 10 x buffer with MgCl2, 20ng of total genomic DNA, 0.25 mM dNTPs (Promega), 100 µM of primers, 1.5mM MgCl2 and 1U of Taq polymerase. Amplification was carried out in thermocycler (MJ Research, USA, Model PCT-200), one cycle of 1 min at 94°C followed by 44 cycles, each consisting of a denaturation step for 1min at 94°C, followed by an annealing step for 1min at 36°C and an extension step for 2 min at 72°C, followed by a final extension step for 5 min at 72°C. After the final cycle the samples were cooled to 4°C. Samples of 10 µl RAPD-PCR product were analyzed by electrophoresis on 1.4% agarose gel and the amplified products were detected under UV light after staining by ethidium bromide. Forty 10-mer primers (Table 1), corresponding to kit A, B, C, D, T, W and Z, were used to study the taxonomy of *Mentha* species.

Table 1. Primers names and their sequences used for *M. spicata*, *Z. tenuior* and *M. longifolia* species in this study.

Primer name	Sequence 5'-3'	Primer name	Sequence 5'-3'
OPA16	AGCCAGCGAA	OPD10	GGTTCACACC
OPA18	AGGTGACCGT	OPD11	AGCGCCATTG
OPA20	GTTGCGATCC	OPD12	CACCGTATCC
OPB01	GTTTCGCTCC	OPD14	CTTCCCCAAG
OPB04	GGACTGGAGT	OPD16	AGGGCGTAAG
OPB05	TGCGCCCTTC	OPD18	GAGAGCCAAC
OPB08	GTCCACACGG	OPD20	ACCCGGTCAC
OPB09	TGGGGGACTC	OPT03	TCCACTCCTG
OPB10	CTGCTGGGAC	OPT05	GGGTTTGGCA
OPB12	CCTTGACGCA	OPT10	CCTTCGGAAG
OPB13	TTCCCCCGCT	OPT13	AGGACTGCCA
OPB14	TCCGCTCTGG	OPT15	GGATGCCACT
OPB17	AGGGAACGAG	OPT16	GGTGAACGCT
OPB19	ACCCCCGAAG	OPT19	GTCCGTATGG
OPC09	CTCACCGTCC	OPT20	GACCAATGCC
OPC10	TGTCTGGGTG	OPW04	CAGAAGCGGA
OPC12	TGTCATCCCC	OPW17	CTCCTGGGTT
OPC20	ACTTCGCCAC	OPZ12	TCAACGGGAC
OPD04	TCTGGTGAGG	OPZ15	CAGGGCTTTC
OPD06	ACCTGAACGG	OPZ16	TCCCCATCAC

2.4. Data analysis

RAPD bands were manually scored as present (1) or absent (0) for the estimation of the similarity among all the tested samples. A matrix of similarity (Jaccard) and similarity of coefficients (Nei and Li, 1979) were calculated and a dendrogram was obtained by clustering according to the Unweighted Pair-Group Method with Arithmetic averages (UPGMA) using SPSS (V., 11.0) software. Polymorphism percentage was estimated by dividing the number of polymorphic bands over the total number of bands.

3. Results

From 40 initially applied primers, only 16 showed reproducible fragments with easily recordable bands. The total number of bands, the number of polymorphic bands along with the percentage of polymorphism are shown in Table 2. A total of 2001 RAPD fragments were consistently recognized, of which 419 were polymorphic in all the tested samples (Table 2). High percentages of polymorphism (26%) showed by OPB01 and OPT16, 26% by OPD06 and OPT15 and 25% for OPT20 (Table 2).

The number of bands varied in different samples with levels of similarity between the samples ranging between 0.68 to 0.03 (Table 3). The highest average similarity index value of 0.68 was observed between two samples (11 and 12) of *M. spicata*. The dendrogram was produced for *Mentha* species and samples showed three main clusters (Figure 1). The first cluster consisted of individuals numbered from 1-10 of *Z. tenuior*. The second cluster consisted of individuals numbered from 11 to 20 samples of *M. spicata*. The third cluster included the samples of *M. longifolia* from 21 to 30. The level of similarity between *Mentha* species and *Z. tenuior* species ranged from 0.68 to 0.02 (Table 3). *Z. tenuior* showing a range of similarity with *M. spicata* and *M. longifolia* (0.21 to 0.06) and (0.13 to 0.03), respectively. On the other hand, genetic variability within each species was found, which is obvious through the presence of sub-clusters within each cluster (Figure 1).

Table 2. Total bands, number of polymorphic bands, percent polymorphism and maximum and minimum number of bands per primer of most polymorphic RAPD primers used among *M. spicata*, *Z. tenuior* and *M. longifolia* in this study.

Primer name	Total bands/primer	Number of polymorphic bands	% of polymorphism	Max./ Min. band per primer
OPB01	99	29	29	6/2
OPB06	140	22	16	6/1
OPB08	99	20	20	5/1
OPB09	124	26	21	10/1
OPB10	113	19	17	6/2
OPB19	145	27	19	7/2
OPD06	109	28	26	7/1
OPD10	171	25	15	10/2
OPD14	186	37	20	9/2
OPD16	166	32	19	8/4
OPT03	133	29	22	8/2
OPT05	116	22	19	6/2
OPT10	122	28	23	8/3
OPT15	113	29	26	9/1
OPT16	88	26	29	5/1
OPT20	77	19	25	5/1
Total bands	**2001**	**419**	**Mean: 22.6**	

4. Discussion

Due to their medical benefits, a very high percentage of the world's population relies on medicinal and aromatic plants (Lawrence, 2007). *Mentha* species are resources for essential oils enriched in certain monoterpenes and are widely used in food, flavor, cosmetic, and pharmaceutical industries (Bhat *et al*., 2002). The following primers OPB01 and OPT16, OPD06 , OPT15, OPT16 and OPT20 showed the highest levels of polymorphism 29%, 29%, 26%, 26% and 25%, respectively, and can be used for further testing of the rest of *Mentha* species 'in Jordan' with molecular and biochemical association.

In this study, *M. spicata, M. longifolia* and *Z. tenuior* formed the three different clusters indicating that each species has a unique DNA sequence, and that a genetic variability exist among them. This result is in agreement with the findings of Mustafa and Bader (2005) who reported that the difference among species could be related to the variants in the alleles numbers between *Mentha* species, and it may be more obvious in the asexual plants *M. longifolia*. The genetic variability, found among the species, could be due to out-breeding and the wide dispersal of seeds and pollen grains. The genetic variation between *Mentha* species can also be explained by the differences in chromosomes numbers (2n=2x= 24) and (2n=2x= 48) in *M. longifolia* and *M. spicata*, respectively (Lawrence, 2007; Murray, 1960). Divergence between *M. longifolia* and *M. spicata* could be a reflection of the impact of environmental variation among the samples of *Mentha* species. This result was in accordance with the results obtained by (Mustafa and Bader, 2005).

In addition to genetic variations, the results of this study indicate that each species has different morphological and biochemical characteristics. Molecular analysis is considered one of the best methods of studying molecular taxonomy to identify and differentiate between species. The findings of the present investigation will be helpful for traditional healers, the local community and all those involved in the study of ethnomedicine, and for scientists to further test these systems. Cultivation should be oriented in the future for essential oil production of *Mentha* species and *Z. tenuior*. Further studies, including the morphological traits, cellular biochemical, molecular data, isozyme polymorphism and karyotyping, should be taken into consideration in the future.

Table 3: RAPD similarity matrix based on similarity coefficient of the amplified bands for *Ziziphora tenuior*, *Mentha spicata* and *Mentha longifolia* collected from different regions in Jordan.

	Ziziphora tenuior										*Mentha spicata*										*Mentha longifolia*									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1.0																													
2	0.41	1.0																												
3	0.46	0.44	1.0																											
4	0.35	0.40	0.53	1.0																										
5	0.30	0.27	0.36	0.35	1.0																									
6	0.30	0.30	0.38	0.40	0.46	1.0																								
7	0.36	0.30	0.41	0.40	0.52	0.47	1.0																							
8	0.24	0.24	0.31	.33	0.39	0.35	0.49	1.0																						
9	0.21	0.21	0.026	0.29	0.41	0.35	0.43	0..51	1.0																					
10	0.22	0.17	0.28	0.30	0.32	0.30	0.37	0.39	0.52	1.0																				
11	0.13	0.13	0.17	0.16	0.16	0.15	0.17	0.17	0.20	0.21	1.0																			
12	0.11	0.12	0.14	0.14	0.14	0.14	0.16	0.15	0.20	0.21	0.68	1.0																		
13	0.16	0.12	0.16	0.15	0.16	0.15	0.14	0.14	0.18	0.20	0.64	0.53	1.0																	
14	0.10	0.10	0.13	0.12	0.14	0.15	0.12	1.14	0.15	0.14	0.64	0.53	0.59	1.0																
15	0.10	0.11	0.14	0.16	0.12	0.11	0.11	0.13	0.15	0.14	0.43	0.39	0.44	0.42	1.0															
16	0.10	0.09	0.10	0.11	0.10	0.08	0.09	0.12	0.13	0.10	0.43	0.43	0.48	0.45	0.53	1.0														
17	0.11	0.12	0.14	0.14	0.15	0.15	0.13	0.17	0.18	0.18	0.42	0.42	0.47	0.42	0.49	0.47	1.0													
18	0.09	0.06	0.09	0.09	0.11	0.10	0.09	0.12	0.15	0.15	0.44	0.39	0.46	0.46	0.44	0.47	0.60	1.0												
19	0.09	0.12	0.11	0.09	0.08	0.07	0.11	0.09	0.10	0.08	0.18	0.15	0.21	0.16	0.22	0.21	0.19	0.16	1.0											
20	0.06	0.08	0.10	0.09	0.08	0.08	0.07	0.09	0.10	0.09	0.14	0.12	0.16	0.14	0.16	0.16	0.15	0.11	0.37	1.0										
21	0.08	0.09	0.11	0.08	0.05	0.08	0.07	0.08	0.07	0.06	0.08	0.08	0.12	0.09	0.17	0.14	0.13	0.07	0.23	0.16	1.0									
22	0.10	0.12	0.13	0.08	0.11	0.11	0.08	0.08	0.10	0.11	0.13	0.11	0.14	0.12	0.15	0.13	0.13	0.10	0.17	0.10	0.31	1.0								
23	0.09	0.10	0.11	0.10	0.08	0.07	0.08	0.07	0.08	0.08	0.09	0.09	0.07	0.08	0.13	0.13	0.12	0.08	0.13	0.10	0.20	0.25	1.0							
24	0.09	0.08	0.10	0.07	0.09	0.08	0.07	0.06	0.07	0.07	0.11	0.10	0.14	0.09	0.11	0.10	0.09	0.10	0.12	0.08	0.17	0.16	0.25	1.0						
25	0.07	0.05	0.07	0.05	0.04	0.05	0.05	0.07	0.06	0.07	0.09	0.06	0.10	0.07	0.08	0.11	0.10	0.10	0.07	0.06	0.23	0.16	0.21	0.36	1.0					
26	0.07	0.07	0.10	0.07	0.04	0.08	0.08	0.08	0.03	0.09	0.08	0.07	0.08	0.06	0.07	0.07	0.07	0.05	0.09	0.09	0.16	0.15	0.20	0.19	0.23	1.0				
27	0.07	0.07	0.11	0.12	0.10	0.09	0.08	0.14	0.07	0.09	0.09	0.08	0.09	0.07	0.12	0.010	0.09	0.08	0.09	0.06	0.16	0.16	0.17	0.28	0.23	0.25	1.0			
28	0.10	0.09	0.10	0.09	0.12	0.09	0.09	0.08	0.08	0.08	0.10	0.11	0.08	0.09	0.10	0.12	0.09	0.08	0.11	0.08	0.15	0.12	0.23	0.19	0.16	0.23	0.19	1.0		
29	0.09	0.09	0.08	0.08	0.11	0.09	0.08	0.05	0.06	0.06	0.09	0.12	0.12	0.10	0.14	0.12	0.11	0.10	0.09	0.05	0.17	0.19	0.21	0.24	0.16	0.22	0.23	0.39	1.0	
30	0.09	0.05	0.12	0.10	0.10	0.07	0.06	0.08	0.08	0.11	0.08	0.10	0.09	0.08	0.11	0.09	0.10	0.08	0.09	0.07	0.14	0.21	0.24	0.26	0.21	0.24	0.28	0.23	0.29	1.0

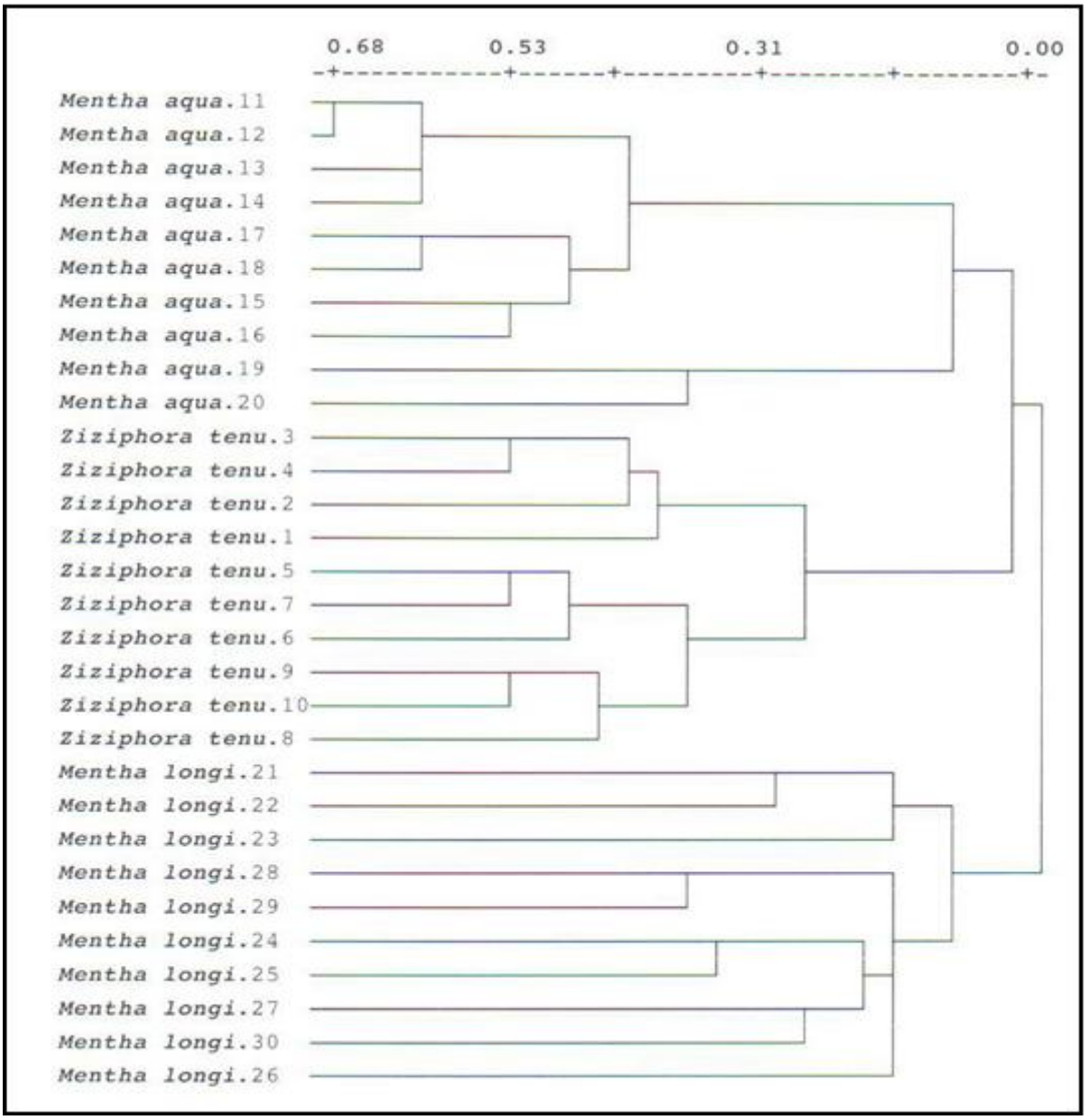

Figure 1. A dendrogram of M. spicata, Z. tenuior and M. longifolia genotypes using sixteen polymorphic RAPD primers, based on Jaccards´ coefficient of similarity.

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Prevalence of *Helicobacter Pylori* Gastritis at the North of Jordan

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Abstract

Helicobacter pylori was isolated from different gastric patients at the north of Jordan. Cultural and histological studies revealed a positive *H. pylori* infection in 78% of the collected samples. Clinical diagnosis showed that 21.6% of *H. pylori* patients were suffering from gastroduodenitis. Histological examination of collected mucosa showed that 67% of *H. pylori* positive patients were having acute and chronic gastritis, whereas 18.3% and 15% of them were suffering from intestinal metaplasia and atrophy, respectively. So, the highest specificity was 84% which was seen in histology results compared to microscopy. However, 58% of infected persons were males and the highest incidence of infection was found in the age 25-35 years old. Isolated *H. pylori* cells were found sensitive to tetracycline, amoxicillin and clarithromycin with an MIC of 0.15, 0.12 and 0.015 µg/ml, respectively.

Keywords: Epidemiology, Gastritis, *Helicobacter pylori*, Jordan.

1. Introduction

Helicobacter pylori is recognized as one of the most common chronic bacterial infections affecting humans worldwide (Rauws *et al.,*1988; Petersen and Krogfelt, 2003).

Infection of *Helicobacter pylori* is highly associated with the upper gastrointestinal tract such as duodenal and gastric ulcers, gastric adenocarcinoma and non-Hodgkin's lymphomas of the stomach (Martin, 1997; Peek and Crabtree, 2006). Duodenal ulcer occurs among persons infected with *H. pylori* which might contribute to chronic atrophic gastritis development which is considered a risk factor for adenocarcinoma of the stomach (Martin, 1997). The role of *H. pylori* gastritis in ulcerogenesis and carcinogenesis was reported by Solcia *et al*. (1994). The most important virulence factors in *H. pylori* disease are believed to be: it's motility, mucinase activity, urease production, adherence factors, heat-labile cytotoxins, hemolysin and lipopolysaccharide, in addition to it's glycocalyx (Figura *et al.*,1989; Geis *et al.*,1989; Dunn *et al.*,1990; Daw *et al.*,1991; MacColm *et al.*,1994; Patrick *et al.*,1994; Petersen and Krogfelt, 2003).

Eradication of the pathogen can be achieved by triple regiment comprising bismuth, metronidazole and an antibiotic such as tetracycline or penicillin (Logan *et al.*, 1991). If metronidazole resistant strains are present, eradication of the pathogen can be achieved with omeprazole and amoxicillin or bismuth and ciprofloxacin. .Monotherapy with clarithromycin was found effective (Logan *et al*., 1991; Stenstrom *et al.*,2008). This study reports the incidence of *H. pylori* gastritis at the north of Jordan.

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2. Materials and Methods

2.1. Sample collection and preparation

Two biopsy specimens each were taken from sixty patients suffering from gastritis and referred for gastroscopy at the endoscopy unit at princes Basma hospital-north of Jordan. At least one of the biopsy specimens was taken from the corpus or the antrum or corpus and antrum of the patient's stomach. All biopsy specimens were taken from patients who had not been treated with bismuth compounds, antibiotics, H_2-receptor blockers or proton pump inhibitors but who showed gastrointestinal illness.

Specimens were collected in brucella broth containing 0.5% bovine serum albumin. They were transported in an ice box to the laboratory for immediate testing and culturing.

2.2. Organism and growth conditions

Biopsy specimens were removed from transporting medium using sterile forceps and 100 µl transport medium was added to the tissue. Then they were ground in a glass tissue grinder and inoculated into blood agar base supplemented with 7% human or horse blood, to which the following antibiotics were added: 10 mg/l vancomycin, 6mg/l amphotericin B and 5 mg/l trimethoprim (Sandra *et al.,* 1999). Mueller-Hinton agar was used to support the growth of *H. pylori* after the addition of 10% fetal calf serum. Incubation was done at 37ºC under microaerophilic environment (BBL Campypack 71034) inside an anaerobic CO2 jar for up to 7 days.

2.3. Identification of H . pylori

Morphological, cultural and biochemical characteristics of *H. pylori* were carried out according to Cliodna and

Julie, 1987; Natale *et al.*, 1989 and Leunk and Johnson, 1988.

2.4. Histological examination

All clinical specimens were processed for histopathological examinations using hematoxylin and eosin stain and Giemsa stain as described by Albertson *et al.*, 1998.

2.5. Statistical analysis

Results of diagnostic techniques were statistically compared using Chi-square analysis.

3. Results

Biopsy samples from sixty patients suffering from gastritis were collected. 62% of patients were males and 38% females, ranging from 23 to 94 years of age. All biopsy specimens were tested using microscopical, cultural and histological methods.

Out of the sixty patients, 47 gave positive cultures of *H. pylori* and the organism was isolated from both antral and corpus biopsies from 53 % of these positive patients (Table 1). Twenty four patients showed positive microscopical examination for *H. pylori*.

Clinical diagnosis showed that 21.6% of *H. pylori* patients were suffering from gastroduodenitis (Table 2). .However, 15% of these patients developed gastric and duodenal ulceration while, 16.6% of *H. pylori* positive patients were diagnosed with atrophic gastritis (Table 2).

Histological examination of patient's mucosa showed three different abnormalities: Acute-chronic gastritis (neutophilic and lymphocytic infiltration), intestinal metaplasia (replacement of gastric mucosa with intestinal mucosa) and gastric atrophy (thinning of gastric mucosa, loss of glandular tissues, and loss of parietal cells). As presented in Table3, 67% of *H. pylori* positive patients were having acute and chronic gastritis, whereas 18.3% and 15% of them were suffering from intestinal metaplasia and atrophy respectively.

Biopsy speciemens showed polymorphpnuclear and round cell infiltration (Fig1a). However, *H. pylori* was shown to colonize the gastric antrum cells (Fig1b).

The highest incidence of *H. pylori* among ages was those ranging from 25-35 years compared to other ages as shown in Figure 2. Isolated *H. pylori* cells were found sensitive to tetracycline, amoxicillin and clarithromycin, when tested using an agar well diffusion method with an MIC of 0.15, 0.12 and 0.015 µg /ml, respectively.

4. Discussion

The prevalence of *Helicobacter pylori* differs significantly both between and within countries, with high rates of infection being associated with low socioeconomic status and high densities of living. (Goodman and Cockburn, 2001; Hazel and Francis, 2002). Approximately, 40 and 80% of adult individuals in developed and developing countries are infected respectively (Timothy and Martin, 1995). However, the percentage of infected people increases with age, since 50% of infected persons were those over the age of 60 compared with around 10% between 18 and 30 years (Pounder and Ng, 1995). But this was not the case in this study, since the highest percentage of patients was among young people ranging from 25-35 years old (Fig 2). In a large French cross-sectional study, a significantly lower prevalence of *H. pylori* infection was observed in females as compared with males (Broutet *et al.*, 2001). However, in this study a highest range of infection was found among males as shown in Figure 2.

In this study 78% of symptomatic patients were infected with *H. pylori*. The infection was associated with variable gastrointestinal illness, chronic gastritis, intestinal metaplasia and atrophic gastritis (Table 2). This is in agreement with others who reported that chronic superficial gastritis associated with *H. pylori* infection is a significant predisposing factor for the development of peptic ulcer, atrophic gastritis, gastric lymphoma and gastric adinocarcinoma (Martin, 1997; Alberto and Mario, 1998).

The highest specificity was 84% which was seen in histology results compared to microscopy (Table 1) which is comparable with Simor *et al.*,(1990) . Isolated *H. pylori* was found sensitive to clarithromycin, tetracycline and amoxicillin and their MICs were comparable to others findings (Pavicic and Namavar1993; Alistair, 1997). A follow-up incidence of *H. pylori* among different ages for the following years will be of importance.

Table 1. Statistical comparison between the three techniques used in the diagnosis of *Helicobacter pylori.*

Test kind	P_{value}	Sensitivity	Specificity	False positive	False negative
Microsc.-Culture	0.031	91.7%	30.6%	8.3%	69.4%
Histology-Culture	0.028	87.2%	53.8%	12.8%	46.2%
Histology-Microscopy.	0.031	46.8%	84.6%	53.2%	15.4%

Table 2. Clinical diagnosis of *H. pylori* positive patients after endoscopy.

Diagnosis	**1***	**2**	**3**	**4**	**2+3****	**3+4**	**2+4**
% *H. pylori* positive patients	10	15	21.6	16.6	25	6.8	5
(Number of patient)	(6)	(9)	(13)	(10)	(15)	(4)	(3)

*1 gastritis, 2 gastric and duodenal ulceration, 3 gastroduodenitis, 4 atrophic gastritis .

**,case repetition and percentage values to be considered.

Table 3. Histological results of *H. pylori* positive biopsy specimens.

	1- Acute and chronic gastritis	**2-Intestinal metaplasia**	**3-Gastric atrophy**
H. pylori positive patients	66.7%	18.3%	15%
(No. of patients)	(40)	(11)	(9)

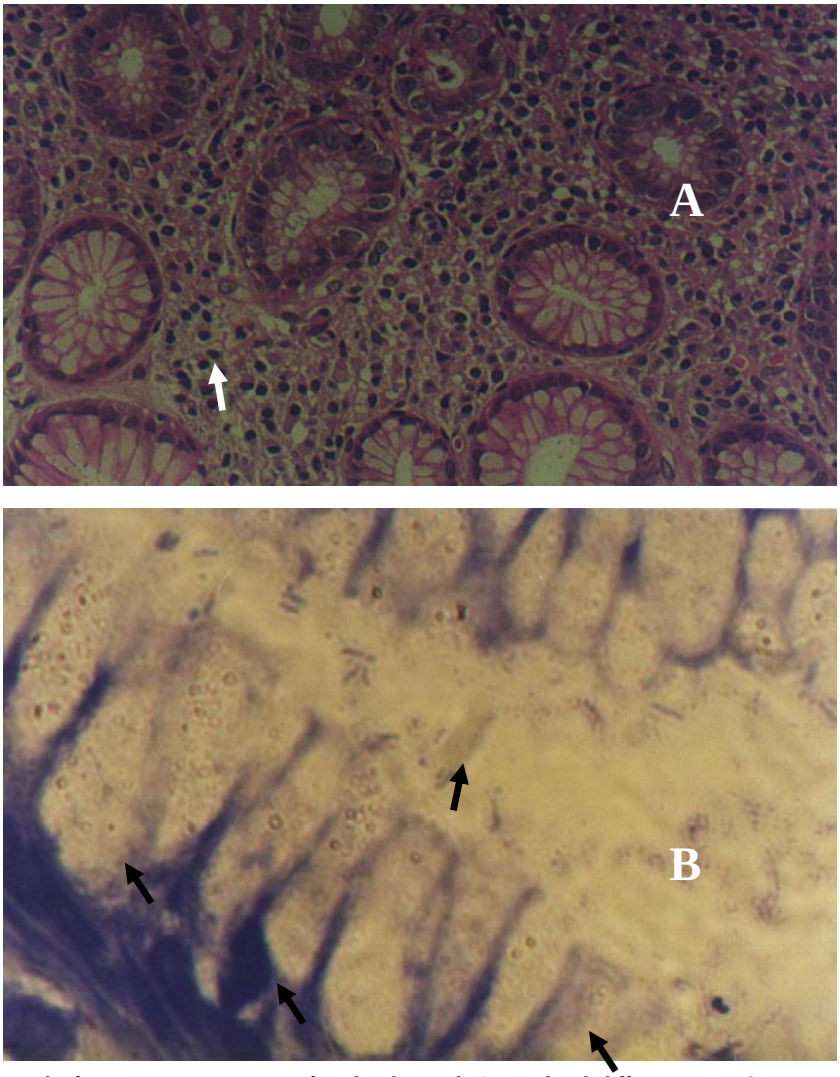

Figure 1. Photomicrograph of a patient gastric antrum infected with *H. pylori* stained with different stains . **A**, Hematoxylin and eosin stain, X 100 : **B**, modified Giemsa ,X 100. Arrow indicates *H. pylori* cells.

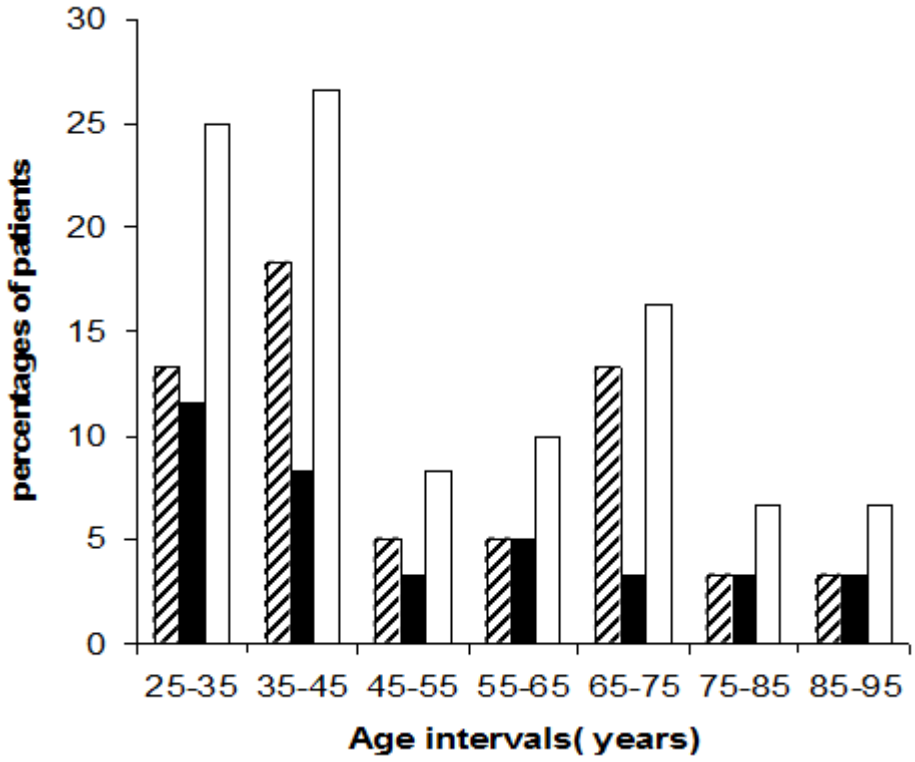

Figure 2. Distribution of *H. pylori* infection among different ages and sexes Male ,■ Female, ⊔ Total.

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Determination of Genetic Relationship among Some Varieties of Chickpea (*Cicer arietinum* L) in Sulaimani by RAPD and ISSR Markers

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Abstract

The molecular evaluation of five chickpea (*Cicer arietinum* L.) varieties [Rania, Chamchamal, Sangaw, FLIP98-133c (screened for their very sensibility to *Ascochyta rabiei*) and FLIP83-48c (screened for their resistance to *Ascochyta rabiei*)] at the University of Sulaimani, College of Agriculture, in 2009-2010 was conducted to assess the genetic diversity and relationship of chickpea genotypes using RAPD and ISSR markers. Five primers of RAPD and ISSR were used of which all primers gave amplification products. On average, 5.8 bands per primer were observed by RAPD and 6.6 bands per primer by ISSR markers. In RAPD, the varieties shared 55.17% polymorphic bands, whereas they shared 63.63% polymorphic bands in ISSR analysis. Cluster analysis by RAPD and ISSR markers revealed clear distinct diversity between genotypes. Rania and Chamchamal showed more similarity than others varieties according to the RAPD data. FLIP83-48c showed the highest dissimilarity comparing with the other varieties. In ISSR analysis, Chamchamal and Sangaw showed more similarity than others varieties. Rania revealed the highest dissimilarity comparing with the others of varieties. Combination of RAPD and ISSR data shared that FLIP83-48c showed the highest dissimilarity comparing with the other varieties. The results showed that ISSR and RAPD analysis for diversity can provide practical information for the management of genetic resources in chickpea breeding program.

Keywords: Chickpea, RAPD, ISSR, Genetic diversity.

1. Introduction

Chickpea (Cicer arietinum L.), as the third most important cool season food legume in the world after dry beans and peas (FAO, 2006), is a diploid, with 2n = 2x = 16 (Arumuganathan *et al.,* 1991) and has a genome size of approximately 931 Mbp. Moreover, chickpea pod covers and seed coats can also be used as fodder. In grain legumes, proteins are an important seed component and are responsible for their relevant nutritional a socio-economic importance. The chickpea seed is a good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight (Talebi *et al.,* 2008). Two main types of chickpea cultivars are grown globally kabuli and desi, representing two diverse gene pools. The knowledge of genetic diversity is a useful tool in gene-bank management and breeding experiments like tagging of germplasm, identification and/or elimination of duplicates in the gene stock and establishment of core collections Genetic diversity among the parents is a prerequisite to improve the chances of selecting better segregates for various characters (Dwevedi *et al.,* 2009).

Differences between genotypes with regard to agronomic, morphological, biochemical (e.g. storage proteins, isozymes), and molecular characteristics are either indirect or direct representations of differences at the DNA level and are therefore expected to provide information about genetic relationships. The assessment of genetic diversity is important not only for crop improvement but also for efficient management and conservation of germplasm resources. For this purpose 5 varieties of chickpea were analyzed by using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers.

Polymerase chain reaction (PCR) method, using arbitrary primers, has been widely utilized in the last 20 years. DNA markers have proved valuable in crop breeding, especially in studies on genetic diversity and gene mapping. The commonly used PCR-based DNA marker systems are RAPD, ISSR, amplified fragment length polymorphism (AFLP) and more recently simple sequence repeats (SSRs) or microsatellites (Gupta *et al.,* 2000). The major limitations of some of these methods are high cost of AFLP and the need to know the flanking

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sequences to develop species specific primers for SSR polymorphism.

The RAPD technique, based on the PCR, is one of the most commonly used molecular markers. RAPD markers are amplification products of anonymous DNA sequence using single, short and arbitrary oligonucleotide primer; thus, they do not require prior knowledge of DNA sequence. Low expense efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable (Bardakci, 2001). RAPD identification techniques can be used at any stage of plant development and they are not affected by environment factors (Lisek *et al.*,2006). The reproducibility of the RAPD techniques can be influenced by variable factor, such as concentration of MgCl2, DNA template; DNA polymerase (Iqbal *et al*., 2002); number of primer; primer sequence; number of PCR cycles (Nkongolo *et al*., 2002) and annealing temperature (Schiliro *et al*., 2001).

ISSR-PCR is a technique overcomes most of these limitations (Zietkiewicz *et al*., 1994). It is rapidly being used by the research community in various fields of plant improvement (Godwin *et al.*, 1997). The technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species.

The aim of this study is to evaluate the genetic diversity of chickpea varieties by RAPD and ISSR markers.

2. Materials and Methods

2.1. Plant material

Five varieties of chickpea including Rania, Chamchamal, Sangaw, FLIP98-133c (screened for their very sensibility to Ascochyta rabiei) and FLIP83-48c (screened for their resistance to Ascochyta rabiei) were used in this study (Table 1). All varieties were obtained from Sulaimani Agricultural Research Center, Sulaimani, Iraq. Healthy seeds with identical dimensions were selected by visual observation.

2.2. Genomic DNA extraction and purification

Seeds were planted in a pot for three weeks at University of Sulaimani, College of Agriculture. Watering was done once a day and, after three weeks, healthy leaves were harvested. Total DNA was extracted from three weeks young chickpea leaves following the CTAB procedure (Cingilli *et al*., 2005).

2.3. RAPD analysis

Eight primers were used in this study, only five primers gave the products (Table 2). The reaction mixture (25 µl) contained 10× assay buffer, 2.5 mM MgCl2, 400 µM dNTP's (Fermantas), 5 pmoles of primer, 100 ng template DNA and 1 U of Taq DNA Polymerase (Fermantas). Amplification was carried out in a thermo-cycler (Master cycler) for 40 cycles, each consisting of a denaturation step at 94 °C for 1 min, annealing at 34 and 36 °C for 50 second and an extension step at 72 °C for 2 min. An initial denaturation step at 94 °C for 5 min, and a final synthesis step of 6 min at 72 °C were also included. Amplification products were separated on 1.5% agarose gel in 1X TAE (Tris base, acetic acid and EDTA) buffer.

2.4. ISSR analysis

PCR amplification was performed as described by Ratnaparkhe *et al.* (1998) with some modifications. Eight primers (UBC primers) were used. Only five primers gave the products (Table 3). Amplification was carried for 40 cycles, each consisting of a denaturation step at 94 °C for 1 min, annealing at 50 °C & 52 °C for 1 min. and an extension step at 72°C for 2 min. An initial denaturation step at 94 °C for 5 min, and a final synthesis step of 6 min at 72 °C were also included. Amplification products were separated on 1.5% agarose gel in 1X TAE buffer.

2.5. Data analysis

Following Lynch and Milligan (Lynch *et al.,* 1994) assumptions, each amplified product was treated as an independent locus and assigned numbers in order of decreasing molecular weight. DNA fragment profiles representing a consensus of two independent replicates were scored in a binary fission with '0' indicating the absence and '1' indicating presence of band. Using the binary data, a similarity matrix was constructed using the Jaccard coefficient (Jaccard, 1908), which was further subjected to clustering analysis and a dendrogram was generated. A cophenetic matrix was constructed using the matrix that was used to generate the clusters. A correlation between the cophenetic matrix and the similarity matrix was determined by using SPSS version 18 (Masumbuko *et al.,* 2003).

3. Results and Discussion

3.1. RAPD analysis

RAPD analysis revealed a good polymorphism among chickpea varieties (Figure 1). Five random primers of RAPD were used in this study. An average of 5.8 bands per primer was observed in a total of 29 bands. From RAPD data 44.83% of common bands and 55.17% (Table 4) of polymorphic bands were observed among chickpea varieties. The primer RAPD-2 and RAPD-4 gave rise to maximum bands (7) and RAPD-3 showed the least number of bands (4).

Cluster analysis was carried out depending on the results of RAPD analysis using the SPSS analysis to find the diversity among the given varieties of chickpea as shown in the dendrogram (Figure 2). At Jaccard dissimilarity of distance 1 Rania and Chamchamal showed more similarity than others varieties.

At distance 20, there are 3 groups: group 1: Rania and Chamchamal, group 2: Sangaw and FLIP98-133c, group 3: FLIP83-48c. At distance 25, Rania, Chamchamal, Sangaw and FLIP98-133c are grouped into one cluster while FLIP83-48c varieties in another cluster. FLIP83-48c showed more dissimilarity distance with the rest of the varieties. The similarity matrix varied from 0.08 to 0.88 in chickpea varieties. The highest value of similarity matrix was registered by Chamchamal and Rania while the lowest value of similarity matrix was recorded by Chamchamal and FLIP83-48c (Table 5). In this investigation, RAPD markers showed a high level of polymorphism and a high number of clearly amplified bands.

The RAPD data reported in this study is in agreement with that obtained by other researchers. Extensive DNA

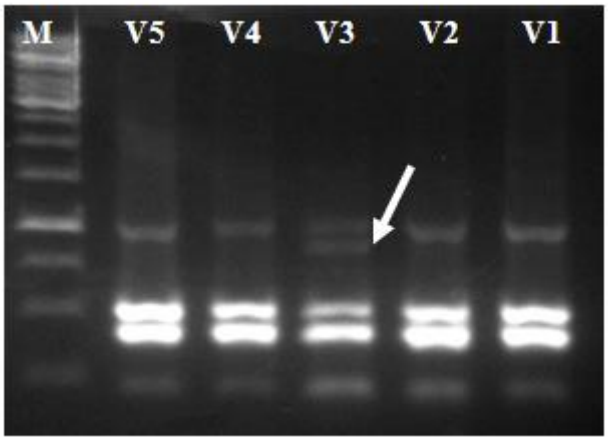

Figure 1. Agarose gel (1.5%) showing the amplified product using RAPD-3 primer. Lane (M): 1 kb DNA ladder, lane (V1): Rania, lane (V2): Chamchamal, lane (V3): Sangaw, lane (V4): FLIP98-133c and lane (V5): FLIP83-48c.

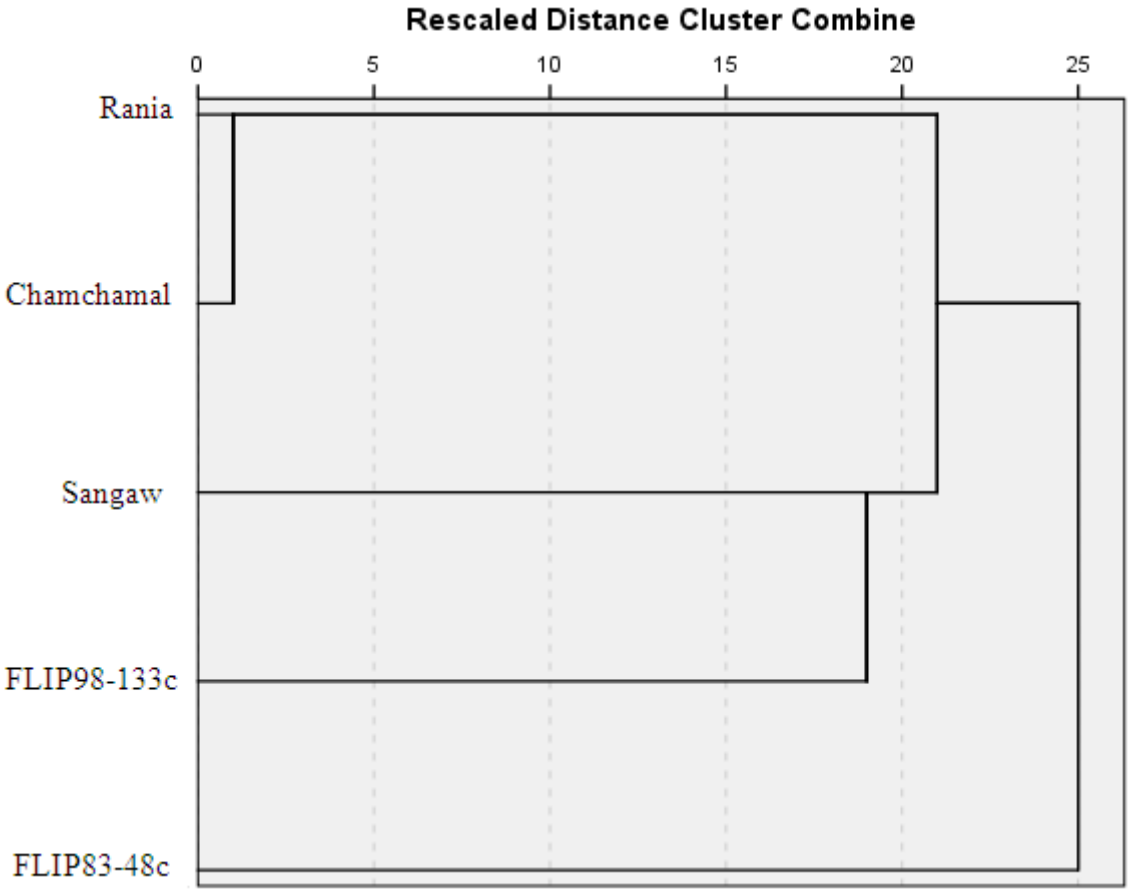

Figure 2. Dendrogram of chickpea varieties showing the genetic similarity based on RAPD data by using cluster analysis.

Table 1. Some agronomic data of chickpea varieties.

Varieties	*Ascochyta rabiei* reaction	Days to maturity
Rania	Susceptible	105
Chamchamal	Susceptible	110
Sangaw	Susceptible	120
FLIP98-133c	Very susceptible	115
FLIP83-48c	Resistant	130

Table 2. RAPD primer with their sequences, percentage of GC and annealing temperature.

Primers	Sequence	GC%	Annealing temperature (^{o}C)
RAPD-1	5'GCGAGTGTG '3	60	36
RAPD-2	5'TCGCTGGTGT '3	60	36
RAPD-3	5'ACAACGCCTC '3	60	36
RAPD-4	5'GGGAACGTGT '3	60	34
RAPD-5	5'GTGATCGCAG '3	60	34

Table 3. ISSR primer with their sequences, percentage of GC and annealing temperature.

Primers	Sequence	GC%	Annealing temperature (^{o}C)
ISSR-1	5' GAAGAAGAAGAAGAAGAA 3'	33	50
ISSR-2	5' ACACACACACACACAC GG 3'	55	50
ISSR-3	5' TGTGTGTGTGTGTGTGAA 3'	44	52
ISSR-4	5' ACACACACACACACACTT 3'	44	52
ISSR-5	5' TGTGTGTGTGTGTGTGGA 3'	50	52

Table 4. Number of amplified fragment, polymorphic fragments, polymorphism percentage, monomorphic fragments and monomorphism percentage based on RAPD data.

Primers	Amplified fragments	Polymorphic fragments	Polymorphism %	Monomorphic fragments	Monomorphism %
RAPD-1	5	3	60	2	40
RAPD-2	7	3	42.85	4	57.14
RAPD-3	4	1	25	3	75
RAPD-4	7	3	42.85	4	57.14
RAPD-5	6	6	100	0	0
Total	**29**	**16**	**55.17**	**13**	**44.83**
Average	**5.8**	**3.2**	**55.17**	**2.6**	**44.83**

Table 5. Jaccard similarity matrix showing the relationship among chickpea varieties based on RAPD data.

	Similarity matrix: Jaccard				
Varieties	**Rania**	**Chamchamal**	**Sangaw**	**FLIP98-133c**	**FLIP83-48c**
Rania	1.00				
Chamchamal	0.88	1.00			
Sangaw	0.31	0.33	1.00		
FLIP98-133c	0.25	0.27	0.33	1.00	
FLIP83-48c	0.15	0.08	0.14	0.27	1.00

polymorphism has been reported using RAPD markers in several other crops (Iruela *et al.,* 2002; Hou *et al.,* 2005). The RAPD-based dendrogram of chickpea genotypes displayed the genetic relationships between these accessions, which accorded with previous studies on chickpea (Ahmad *et al.,* 1992; Tayyar *et al.,* 1996 and Iruela *et al.,* 2002). Although the *Cicer* species are predominantly self-pollinating, more variation was observed among them. The reason for this genetic variation could be that the specific accessions were heterozygous at some marker loci. Similar observations were reported in pea, lentil (Simon *et al.,* 1997), and chickpea (Moussa *et al*., 1996; Sant *et al*., 1999). Iruela *et al.,* 2002) showed that RAPD markers successfully identified genetic variation in *Cicer*. The variation identified was greater than that revealed by the isozymes or seed storage proteins used in previous studies of genetic relationships among annual *Cicer* species (Ahmad *et al.,* 1992; Labdi *et al.,* 1996; Tayyar *et al.,* 1996). Further, large amount of genetic variation which exists between chickpea genotypes can be used efficiently for gene tagging and genome mapping of crosses to introgression the favorable traits such as high yield potential, disease and insect resistance into the cultivated genotypes. Thus, RAPD markers were good indicators of morphological divergence.

3.2. ISSR analysis

The importance and need of chickpea varieties at global level requires evaluation of germplasm to assist the future breeding programs. Hence, it is essential to characterize chickpea germplasm using markers like PCR-based marker such as RFLPs, RAPDs and microsatellites. Five primers (ISSR-1, ISSR-2, ISSR-3, ISSR-4, and ISSR-5) were found to be polymorphic (Figure 3). On an average, 6.6 bands per primer were observed in a total of 33 bands (Table 6). The varieties shared 36.37% common bands and 63.63% polymorphic bands with ISSR markers. Out of five polymorphic ISSR primers, ISSR-2 given the maximum bands (11) and ISSR-3 showed least number of bands (2).

Dendrogram cluster analysis, resulted from ISSR using the SPSS analysis, showed diversity among the given varieties on the bases of similarity matrix of Jaccard. The similarity matrix varied from 0.16 to 1.00 in chickpea varieties (Table 7). The highest value of similarity matrix was registered by Chamchamal and Sangaw while the lowest value of similarity matrix was recorded by Rania and FLIP83-48c. The diagram (Figure 4) revealed four main groups: group 1 includes Chamchamal and Sangaw, group 2 contains FLIP98-133c, group 3 contains FLIP83-48c and group 4 includes Rania. At Jaccard dissimilarity of distance 1, the varieties: Chamchamal and Sangaw showed more similarity than others varieties. At distance 17, there are 3 groups: group 1includes Chamchamal, Sangaw and FLIP98-133c, group 2 contains FLIP83-48c and group 3 contains Rania. The varieties Rania and FLIP83-48c showed more dissimilarity distance with the rest of the varieties.

When compared to the RAPD dendrogram, the ISSR dendrogram showed more correlation with the pedigree data, which shows that the ISSR markers are the most efficient marker system, because of their capacity to reveal several informative bands from single amplification. Similar observations were reported by Bornet and Branchard (2001), Fernandez *et al.* (2002) in barley and Qian *et al.* (2001) in rice. Since ISSR markers are dominant, the similarity at the sequence level of monomorphic bands can be questioned. But numerous studies verified that most co-migrating fragments are identical by descent, at least at the intraspecific level (Wu *et al.,* 2000; Sales *et al.,* 2001). Ratnaparkhe *et al.* (1998) studied the inheritance of Inter-simple sequence repeat polymorphisms and linkage analysis with *Fusarium* resistance gene in chickpea. They demonstrated that a simple sequence repeat (AC) 8YT was linked to the gene for resistance to Fusarium wilt race 4. Rao *et al.* (2007) reported the ISSR fingerprinting in cultivated chickpea and its wild progenitor to correlate the relationship measures based on pedigree data and morphological traits for the selection of good parental material in chickpea breeding programs. Rajesh *et al.* (2003) reported that genetic relationship analysis based on ISSRs supports the morphological and crossability data, ISSRs prove to be an efficient marker system. The diversity thus observed with microsatellites in the chickpea germplasm is probably due to the use of landraces throughout most of the Indian subcontinent (Malhotra *et al.,* 1987; Sant *et al.,* 1999), and even today these landraces are being used for the development of elite cultivars. However, the genetic diversity between the various landraces still remains to be studied and molecular markers will be greatly useful in quantifying this diversity.

3.3. RAPD and ISSR dendrogram

To decrease the inaccuracies of the independent techniques, a dendrogram was developed by pooling the data of both RAPD and ISSR. Two major clusters were observed in this dendrogram (Figure 5). Chamchmal and Sangaw grouped together into one major cluster, whereas

Rania, FLIP98-133c and FLIP83-48c formed the second, third and fourth cluster respectively. The similarity matrix

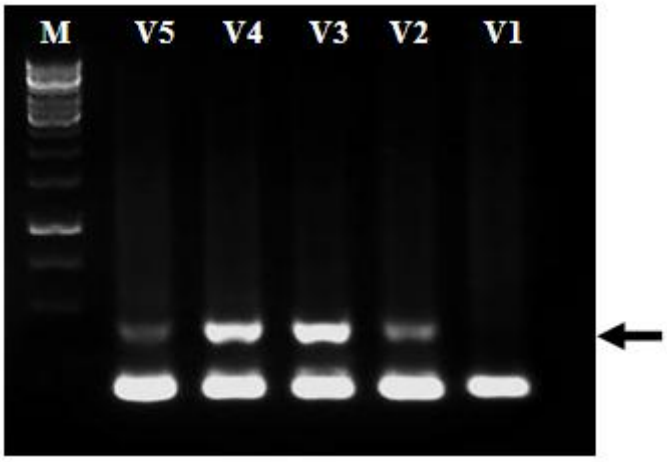

Figure 3. Agarose gel (1.5%) showing the amplified product using ISSR-3 primer. Lane (M): 1 kb DNA ladder, lane (V1): Rania, lane (V2): Chamchamal, lane (V3): Sangaw, lane (V4): FLIP98-133c, and lane (V5): FLIP83-48c.

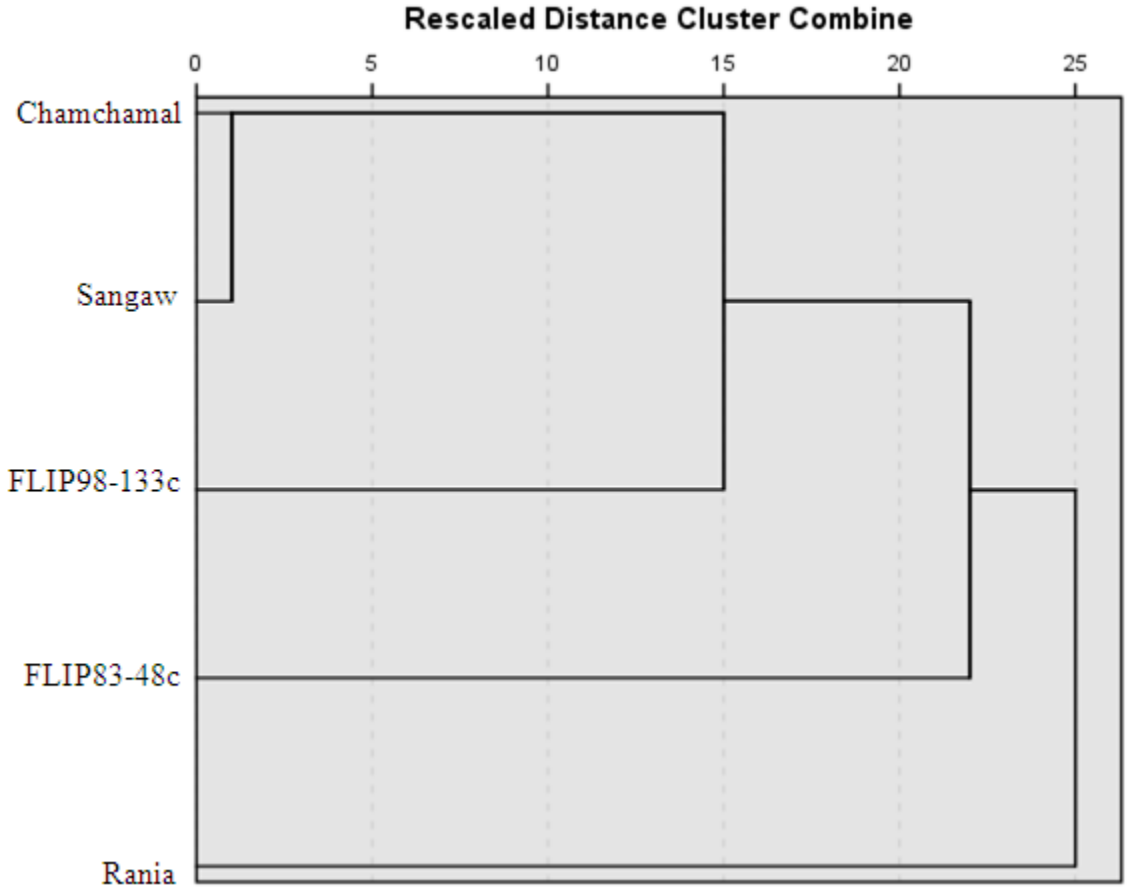

Figure 4. Dendrogram of chickpea varieties showing the genetic similarity based on ISSR data by using cluster analysis.

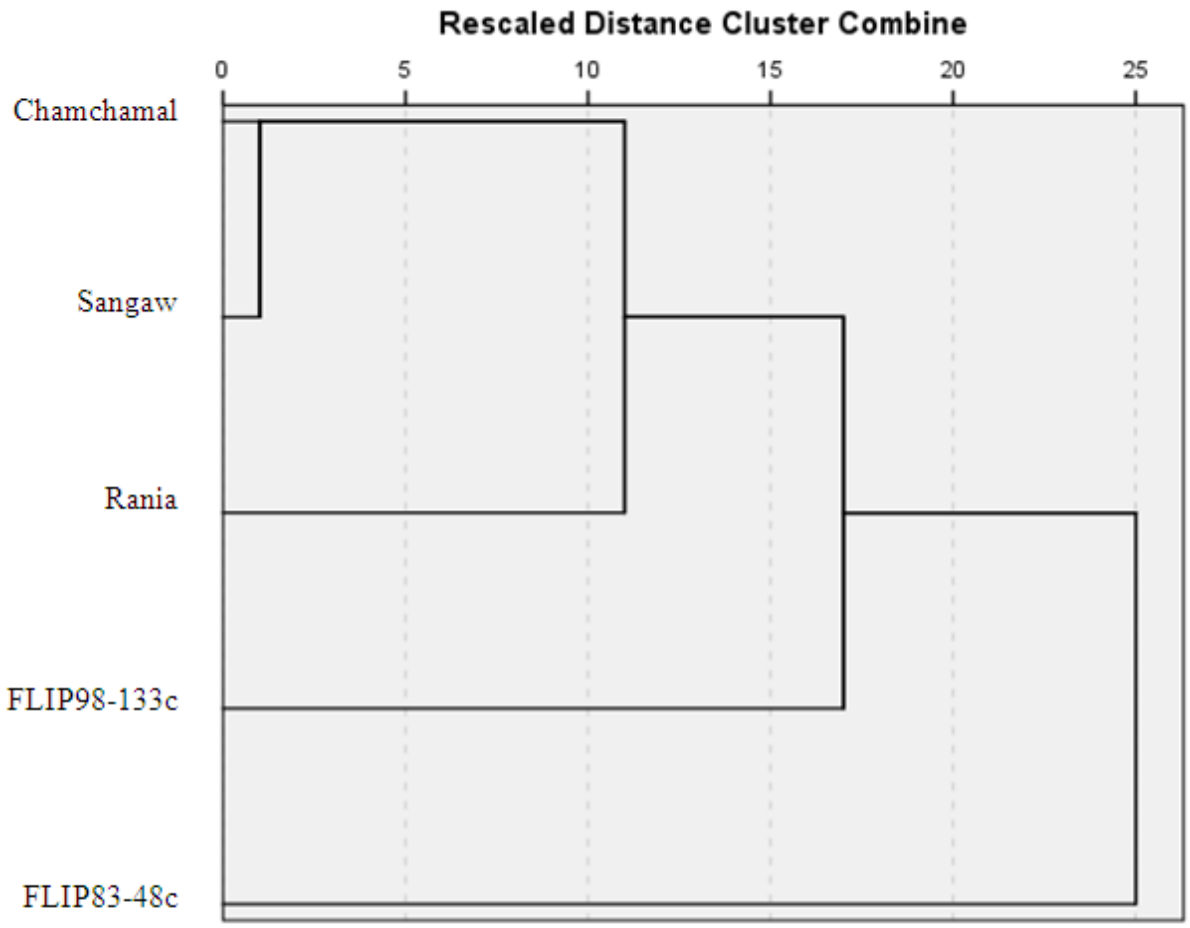

Figure 5. Dendrogram of chickpea varieties showing the genetic similarity based on RAPD and ISSR data by using cluster analysis.

Table 6. Number of amplified fragment, polymorphic fragments, polymorphism percentage, monomorphic fragments and monomorphism percentage based on ISSR data.

Primers	Amplified fragments	Polymorphic fragments	Polymorphism %	Monomorphic fragments	Monomorphism %
ISSR-1	8	5	62.5	3	37.5
ISSR-2	11	7	63.64	4	36.36
ISSR-3	2	1	50	1	50
ISSR-4	6	4	66.67	2	33.33
ISSR-5	6	4	66.67	2	33.33
Total	**33**	**21**	**63.63**	**12**	**36.37**
Average	**6.6**	**4.2**	**63.63**	**2.4**	**36.37**

Table 7. Jaccard Similarity matrix showing the relationship among chickpea varieties based on ISSR data.

	Similarity matrix: Jaccard				
Varieties	**Rania**	**Chamchamal**	**Sangaw**	**FLIP98-133c**	**FLIP83-48c**
Rania	1.00				
Chamchamal	0.44	1.00			
Sangaw	0.44	1.00	1.00		
FLIP98-133c	0.22	0.62	0.62	1.00	
FLIP83-48c	0.16	0.50	0.50	0.25	1.00

Table 8. Jaccard Similarity matrix showing the relationship among chickpea varieties based on RAPD and ISSR data.

	Similarity matrix Jaccard				
Varieties	**Rania**	**Chamchamal**	**Sangaw**	**FLIP98-133c**	**FLIP83-48c**
Rania	1.00				
Chamchamal	0.58	1.00			
Sangaw	0.38	0.65	1.00		
FLIP98-133c	0.23	0.46	0.48	1.00	
FLIP83-48c	0.16	0.30	0.32	0.26	1.00

for the chickpea varieties varies from 0.16 to 0.65 (Table 8), whereas the maximum value of similarity shared by Chamachamal and Sangaw whereas Rania and FLIP83-48c revealed the minimum values. FLIP83-48c showed the highest dissimilarity comparing with the others of varieties.

This observation was consistent with the study of Simon and Muehlbauer (1997), who detected variation within single *C. reticulatum* accession (PI 489777), used to generate an interspecific mapping population. Our results are in accordance with Iruela *et al.* (2002). Iruela reported the genetic diversity among *C. arietinum* varieties using RAPD and ISSR. Shan *et al*. (2005) showed that a natural hybrid could be useful for bridging crosses to introduce genes to chickpea from incompatible species given that *C. reticulatum* was the wild progenitor of chickpea.

ISSR analysis is more economical and reliable than that of RAPD. Earlier studies also reported that ISSR technique generates large number of polymorphisms in chickpea (Collard *et al*., 2003a). The phylogenetic relationship between *Cicer* species from this study was overall consistent with most previous studies (Croser *et al.*, 2003; Nguyen *et al.*, 2004; Sudupak, 2004).

4. Conclusions

The present investigation demonstrates the potential of RAPD and ISSR fingerprinting in detecting polymorphism among chickpea varieties. Varieties FLIP83-48c showed the highest dissimilarity comparing to others varieties. Genetic information obtained from RAPD and ISSR markers can be used in discriminating chickpea varieties and can complement the genetic information generated from the morphological traits. Further, the genetic variation which exists between chickpea varieties can be used efficiently in plant breeding.

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Association of Entomopathogenic and Other Opportunistic Fungi with Insects in Dormant Locations

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Abstract

A survey of entomopathogenic and other opportunistic fungi associated with seven naturally infected insect species live hidden in some plants at their hibernation sites at Gara mountain, Kurdistan region of Iraq was carried out. *Aspergillus flavus*, *A. niger* and *Beauveria bassiana* were detected with high isolation rates. Several other opportunistic pathogens including *Alternaria alternata*, *Curvularia* sp., *Fusarium* sp., *Humicola* sp., *Penicillum* sp., *Rhizopus stolonifer*, *Ulocladium atrum*, *Trichoderma* sp., and sterile mycelium were also isolated. *Beauvaria brongniartii* was isolated from Sunn pest (Eurygoster *integriceps*) for the first time in Iraq. A brief description along with photographs is provided for the newly recorded species.

Keywords: Entomopathogenic fungi, opportunistic fungi, insects, Iraq.

1. Introduction

Fungal diseases of insects are common and widespread and often contribute to the natural regulation of insect populations (Samson *et al.*, 1988; Hajek and Leger, 1994). Entomopathogenic hyphomycetes have great potential as biological control agents against insects and as one component within integrated pest management systems. They are being developed worldwide for the control of many pests of agricultural importance (Ferron, 1985) and some are already available commercially for the control of various species of trips and aphids (Goettel *et al.*, 1990; Upadhyay, 2003).

As regards of insects, especially members of Hemiptera (Family: Scutelleridae and Pentatomide) live from summer to next spring hidden in some plants (e.g. *Acantholinon acerosum* (Willd.) Bioss) and after hibernation they get outside where reproduction proceeds (Paulian and Popove, 1980; Khanjani and Mirab, 2004). During their hibernation, insects are subjected to infection by entomopathogenic and other opportunistic fungi (Kubatove and Dorak, 2005).

In the Iraq Republic, the most common insect species found in dormant locations was *Eurygaster integriceps* Put. (Sunn pest) the highest percentage of its mortality was caused by the entomopathogenic fungus *Beauvaria bassiana* (Bals.)Vuill. (Assaf, 2007). KiliC (1976) showed ability of *B. bassiana* to kill up to 80% of the sunn pest. Comparable 80% mortality was induced by *B. bassiana* and *Fusarium* spp. entomopathogenic fungi associated with *E. integriceps* in dormant locations of Iraq were recorded by Ali (1995). Mohamad (2000) found the highest level of sunn pest infection by *B. bassiana* (80.18%) during October in the Safeen mountain (Irbil province).

The main aim of the current investigation is to extend our knowledge on the occurrence of entomopathogenic and other opportunistic fungi infecting insects in dormancy locations.

2. Materials and Methods

2.1. Insect collection

Sunn pests and other insects (Table 1) were collected from their hibernation sites at Gara mountain , Duhok governorate, North Iraq for two years between October to December of 2009-2010 consecutively. Collected insects were taken to the laboratory in clean bags for isolation of fungi.

2.2. Isolation of fungi

Collected insects were surface sterilized in 2% sodium hypochlorite solution for 3 minutes, rinsed in plenty of sterile distilled water, then dried by filter paper. Surface sterilized cadavers were plated onto potato dextrose agar (PDA) (Himedia Laboratories Pvt. Ltd. - India) containing 0.25 mg/ml chloramphenicol to inhibit growth of bacteria and incubated at 25°C. Hyphae of the fungi growing and sporulating on cadavers and on PDA medium were cut, transferred on fresh PDA plates and incubated at 25°C.

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Table 1. Insects specimens collected from Gare Mountain in the Duhok province-Iraq

Taxonomic group of insects (order, family)	Insect species	Number of Insects
Hemiptera, Pentatomidae	*Dolycoris baccarum* L.	107
Hemipetera, Scutelleridae	*Eurygoster integriceps* Put.	25
Coleoptera, Scarabaeidae	*Anomala* sp.	5
Orthoptera, Acrididae	*Acrotylus insubricus* Scop.	1
Hemiptera, Pentatomidae	*Aelia acuminate* L.	2
Hemiptera, Pentatomidae	*Apodiphus* sp.	3
Coleoptera, Coccinellidae	*Coccinella novemnotata* Herbft	6

2.3. Identification of fungal isolates

Identification of fungal isolates was mainly based on their morphological characteristics of their reproductive structures with the aid of relevant taxonomic keys (de Hoog, 1972; Samson *et al.,* 1988; Tzean *et al.,* 1997; Domsch *et al.,* 1980).

Isolation percentage of a particular species on insect was calculated using the formula:

Isolation percentage = (Number of fungal isolates of a particular species / Total number of isolates of all species) X 100

3. Results

A total of 149 cadavers belong to seven insect species were examined for the presence of fungi. Approximately 226 fungal colonies assigned to 12 different species and sterile mycelium were isolated (Table 2). The highest number of isolates (184 and 25) was detected from *Dolycoris baccarum* and *Eurygoster integriceps*, respectively.

Aspergillus flavus (33.70%), *Aspergillus niger* (26.63%), *Beauveria bassiana* (10.33%) and *Alternaria alternata* (9.78%) were the most common fungal species isolated from *Dolycoris baccarum*, whereas *B. bassiana* showed the highest isolation percentage (36.00%) on *Eurygoster intergriceps*, followed by *Ulocladium atrum* (20.00%) and *Rhizopus stolonifer* (16.00%). Fungi isolated from *Coccinella novemnotata* were in descending order, **B**. *bassiana* (33.33%), *R. stolonifer* (22.22%), followed by *A. alternate*, *A. flavus*, *A. niger* and *Trichoderma* sp. (11.11% each). *Beauveria* was not isolated from the other four insect species. *Beauveria brongniartii* was isolated from the sunn pest *Eurygoster integriceps* for the first time in Iraq. The newly recorded species is described and illustrated.

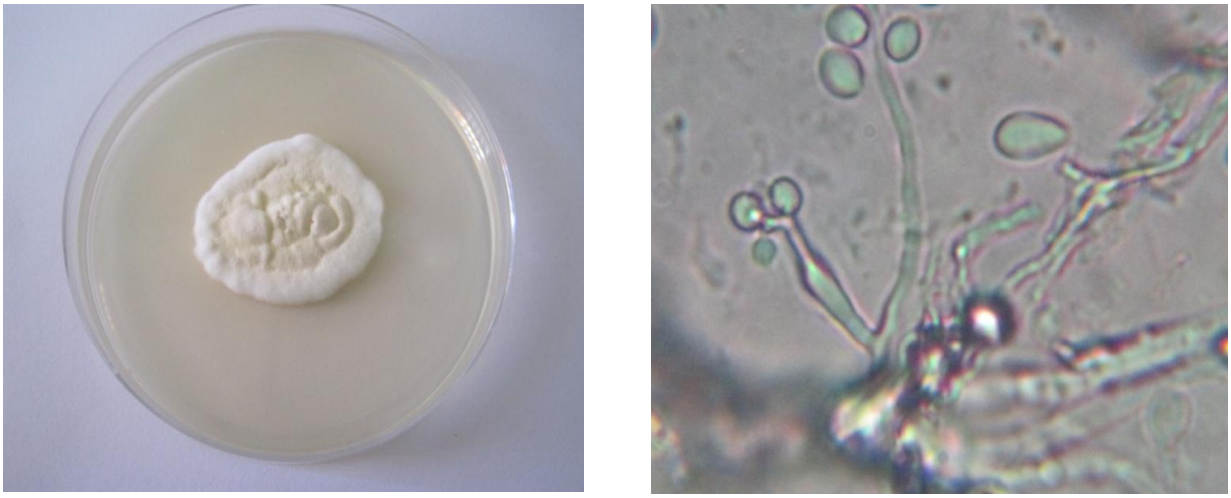
3.1. Phenotypical characterization of Beauvaria brongniartii *(Sacc.) Petch.*

Trans. Br. Mycol. Soc. 10:249.1924. Syll. Fung. 10:540. 1892. Fig. (1) A-B.

Fungal colony on PDA reached a radial of 37 mm after 25 days: floccose, velvety to powdery, at first white, later often becoming yellowish to pinkish. Reverse yellowish to orange. Hyphae hyaline, smooth-walled, 2- 4 µm wide, bearing groups of swollen lateral cells, globose, cylindrical to sub-cylindrical. Conidiogenous cells are arranged in small groups or solitarily along the hyphae consisting of globose to sub-globose basal part 3×3 µm and terminal cell; terminal cells mostly slender, rachis well developed 20 × 2 µm geniculate or irregularly bent, denticulate, denticles thinner than rachis. Conidia oblong to ellipsoidal, hyline, smooth-walled, base slightly apiculate, 2.5–4.8× 2.5–3 µm. Chlamydospores not observed. This description was in agreement with de Hoog (1972) and Tzean *et al.,* (1997).

Table 2. Isolation percentage of fungi and their association with insect species.

Insect species	Associated fungus species	No. of isolated fungi	Isolation percentage (%)
Dolycoris baccarum L.	*Alternaria alternate*(Fr.) Keissl.	18	9.78
	Aspergillus flavus Link	62	33.70
	Aspergillus niger Tiegh.	49	26.63
	Beauvaria bassiana (Bals.) Vuill.	19	10.33
	Curvularia sp.	1	0.54
	Fusarium sp.	1	0.54
	Humicola sp.	2	1.08
	Penicillium sp.	3	1.63
	Rhizopus stolonifer (Ehrenb.) Vuill.	13	7.07
	Sterile mycelium	16	8.70
Eurygoster integriceps Put.	*Aspergillus niger* Tiegh.	2	8.00
	Beauvaria bassiana (Bals.) Vuill.	8	36.00
	Beauvaria brongniartii (Saccardo) Petch	1	4.00
	Penicillium sp.	3	12.00
	Rhizopus stolonifer (Ehrenb.) Vuill.	4	16.00
	Ulocladium atrum Preuss	5	20.00
	Sterile mycelium	2	8.00
Anomala sp.	*Rhizopus stolonifer* (Ehrenb.) Vuill.	1	50
	Penicillium sp.	1	50
Acrotylus insubricus Scop.	*Aspergillus niger* Tiegh.	1	100
Aelia acuminata L.	*Aspergillus flavus* Link	2	100
Apodiphus sp.	*Alternaria alternate* (Fr.) Keissl.	3	27.27
	Aspergillus flavus Link	2	18.18
	Aspergillus niger Tiegh.	1	9.09
	Mucor sp.	2	18.18
	Penecillium sp.	1	9.09
	Rhizopus stolonifer (Ehrenb.) Vuill.	2	18.18
Coccinella novemnotata Herbft	*Aspergillus niger* Tiegh.	1	11.11
	Aspergillus flavus Link	1	11.11
	Alternaria alternata (Fr.) Keissl.	1	11.11
	Beauvaria bassiana (Bals.) Vuill.	3	33.33
	Rhizopus stolonifer (Ehrenb.) Vuill.	2	22.22
	Trichoderma sp.	1	11.11



A B

Figure 1. *Beauvaria brongniartii* . (**A**) Twenty five day old colony on PDA; (**B**) Conidiogenous cells and Conidia. Scale bar of B = 10 µm.

4. Discussion

Most insect-associated fungi found in Iraq during this study have been reported from other parts of the world (Va¨nninen *et al.,* 1989; Va¨nninen, 1995; Meyling and Eilenberg, 2006). A total of 12 species and sterile mycelium were detected in the cadavers of different insects. Regarding entomopathogenic fungal species, *B. bassiana* was the most frequently isolated fungus from three insect taxa and with a relatively high isolation percentage. This result is in agreement with several other studies indicating that *B. bassiana* has a relatively broad host range and is encountered from different ecosystems (Doberski and Tribe, 1980; Vanninen, 1995; Tzean *et al*., 1997; Jankevica, 2004; Kubatova and Dvorak, 2005; Meyling and Elenberg, 2006). *B. bassiana* seems has a wide distribution over the country and has been repeatedly isolated from different insects as well as from different soils in Iraq (Khalaf *et al*.,1997, 1998; Al-Doski, 2007; Abdullah and Mohamed Amin, 2009). The genus *Beauveria* contains a number of species, all of which are pathogenic to insects (Zimmerman, 2007). *B. brongniartii*, a newly recorded in Iraq is detected in one occasion on the sunn pest, *Eurygoster integriceps*. The scarcity of this fungus is largely attributed to its very limited host range and its poor saprophytic competitive ability (Keller *et al.*, 2003: Kessler *et al.,* 2004). The description of our isolate is in agreement with de Hoog (1972). It is very close to *B. bassiana*, but can be separated by its conidial shape and size (de Hoog, 1972: Tzean *et al.*,1997).

Aspergillus flavus and *A. niger* isolated in the present study have previously been found in significant incidence rates on different insect species by several authors (Hermandez-Crespo *et al.,* 1997; Gunde-Cimerman *et al.*, 1998: Balogun and Fagade, 2004). The two species were reported as pathogens to the larvae and pupeae of *Musca domestica* L. under laboratory conditions (Khalaf *et al*., 1997, 1998). These two species were also isolated from populations of the subterranean termite *Microcerotermes diversus* in Basrah, Iraq (Abdullah *et al.,* 2001,2002).

Fusarium sp. was detected from *Dolycoris baccarum* cadaver in one occasion. *Fusarium* species have also been isolated from larvae and adult insects and were reported as insect pathogens (Claydon and Grove, 1984; Sur *et al*., 1999), and as soil opportunistic pathogen to insects in several studies (Ali-Shtayeh *et al.,* 2002; Sun and Liu, 2008; Sun *et al*.,2008; Abdullah and Mohamed Amin,2009).

In this study, several other fungal species including *Alternaria alternata*, *Curvularia* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus stolonifer* , *Trichoderma* sp., *Ulocladium atrum* and sterile mycelium were detected from dead cadavers of different insect species. These species are considered as secondary colonizers unless proven their pathogenicity. However, isolates from the *Mucor*, *Penicillium* and *Trichoderma* genera were considered as opportunistic pathogenic fungi of insects (Gunde-Cimerman *et al*.,1998; Ali-Shtayeh *et al.,*2002, Sun *et al.,*2008 and Abdullah and Mohamed Amin, 2009).

The high isolation percentage of *B. bassiana* and other opportunistic fungi from the cadavers of sunn pests and other insects suggests that probably these fungi display an important role in regulating insect populations of the two important pests during their dormancy at hibernation sites.

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Microbiological Changes and Determination of Some Chemical Characteristics for Local Yemeni Cheese

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Abstract

The changes in microbiological parameters during the storage of local Yemeni cheese were studied. Both the smoked and the non smoked cheese were produced by using traditional techniques in some regions of Yemen. Microbiological examination was carried out at 2, 4 and 7 days of storage for serve cheese and compared with control cheese prepared in laboratory. During storage period, the total viable bacteria count, lactic acid bacteria, staphylococci, coliforms, yeast and molds increased and reached to 11.97, 10.36, 11.8, 13.4, 11.8 and 8.9 Log cfu/g, respectively, in some samples and then decreased at the end of storage period. Also, the number of coliforms, staphylococci, yeast and molds in cheese samples were higher than limits allowed by the national standards for Yemeni soft cheese. Pathogenic flora as *Salmonella* and *Listeria* were detected in some samples and disappeared at the end of storage period. . The hygienic quality of smoked cheese was best than non smoked cheese. At the final days of storage a sharp drop in pH values changing from 5.4 to 4.1 was noticed. The average contents of chemical composition of smoked and non-smoked cheeses were that, moisture 46.55 and 57.27%, fat 21.29 and 20.67 %, protein 14.87 and 16.98% salt 5.01 and 3.75 % respectively.

Keywords: Smoked and non smoked Yemeni cheese, microbiological changes, storage period, chemical composition.

1. Introduction

Smoking of foods is one of the oldest methods of food preservation but, presently, foods are smoked for sensory quality rather than for preservative effect. In general, smoking infuses the high-protein food with aromatic components, which lend flavor and color to the food and also play bacteriostatic and anitioxidant roles (Bratzler *et al*., 1969; Poutler, 1988; Horner, 1992).The most common smoked varieties of cheese are Seretpanir (Iran), Caramakase (Germany), Bandal (India), and Provolone (Italy). In a study at Michigan State University in the 1960s, it was found that smoked cheeses sold at 10 cents per pound more than similar nonsmoked cheeses and increased sales by 45% (Kosikowski and Mistry, 1997).There are reports that phenolic compounds found in smoke inhibit growth of microorganisms on smoked Cheddar cheese (Wendorff *et al.*, 1993). Rheological and fracture properties are of great importance for the producer, the market and the consumer. These properties differ depending on the type of cheese, the stage of maturation and also depending on the composition of the cheese as content of water, fat, salt, pH, protein degradation and environmental factors such as temperature (Walstra and Peleg, 1991).Historically there have been outbreaks of infection associated with the consumption of cheese and the predominant organisms responsible have included *Salmonella*, *Listeria monocytogenes*, Verocytotoxin producing *Escherichia coli* (VTEC) and *Staphylococcus* sp. (Razavilar, 2002; Karim, 2006; Tamagnini *et al*.,2005)

Smoked cheese is the most popular cheese in Yemen. It is made primarily from raw goat's milk by some villagers under unsanitary conditions. The product is considered as a semi-hard cheese with about 40% moisture content and characterized as a salted cheese with an attractive light brown color imposed by smoking (Al-Zoreky, 1998).There is no standardized technique for the manufacture of Smoked cheese, only using traditional methods in the different geographical locations in Yemen without species starters. It is true that it is potentially unsafe and could cause problems in the future if its production conditions are not improved. These types of cheese were marketing during 7 days after production.

The aim of the present study was to evaluate the changes of the main groups of microorganisms during the storage, handling and determination some chemical characteristics of local Yemeni cheese. Also we sought to investigate, the processed smoked cheese in healthy conditions and its comparison to their traditional products.

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2. Materials and Methods

2.1. Cheese making and samples collection

These types of cheese are made from the milk of cows, sheep or goat by some villagers without heat treatment. Clot milk is extracted from the stomach of young goats, which are no older than two weeks to be used as the milk curdles. A small amount of clot milk is added to fresh milk, mixed and left several hours until formation strength curd, then salt is added .The blocks of cheese are exposed to smoking by using types of wood Althahya or Almziz or Alhamer or other woods used for this purpose.

Five samples of fresh cheese processed by a traditional procedure were collected from several local markets in Taiz (samples A and B from Al-bab alkabeer and samples C, D and E (non-smoked) from Albarh). Samples were transported to the laboratory and kept overnight before analyses. Samples L1 and L2 (non-smoked) were prepared in the laboratory of Biotechnology and Food Technology Department, Faculty of Agriculture and Veterinary by a standard protocol (kosikowski and Mistry, 1997). One type of local wood material, most commonly used in cheese smoking, namely Althahya, was used in this study. The curd cheese (L1 and L2) was pressed and divided into small blocks. The blocks of cheese were subjected to heat and wood smoke by placing them on the top grate with suitable space between blocks of cheese and wood. The smoking was continued until the surface of the cheese blocks had a nice brown color all over and imparted a characteristic aroma and flavor.

2.2. Microbiological analysis

All microbiological analysis was performed according to American Public Health Association (APHA, 2002). Cheese samples (10 g) were mixed with 90 ml of warm (40°C) sterile 2% Na citrate and homogenized in a Stomacher for 3 min. Sequential decimal dilutions of the homogenate were prepared in sterile peptone water and plated in duplicates onto specific media. Plate Count Agar (Himedia,India) was used for the total aerobic bacteria count. The petri dishes were aerobically incubated for 24-48 hours at 37°C. Potato dextrose agar of pH 3.5 adjusted with 10% tartaric acid (PDA) (Himedia. India) was used for moulds-yeast counts. MRS Agar (Biolab, UK) was used for the lactic acid bacteria count. All colonies created after incubation at 35°C for 48-72 hours of plates planted with two layers were counted. Violet Red Bile Agar (Himedia, India) was used to coliform count. *Salmonella* detection was carried out after enrichment of sample in Selenit cystine broth (Himedia, India) and incubated at 37 °C for 18-24h.After the enrichment step, the cultures were surface streaked onto Salmonella /Shigella agar (Oxoid, UK) and colorless colonies with black centers were counted after 48h of incubation at 37°C. Enumeration of *Staphylococcus* sp. was performed on Staph. Agar110 (Biolab). Yellow colonies were counted after 24h of incubation at 37°C. For *Listeria* detection each sample (25 g/ml) was taken and placed in a stomacher bag to which 225 ml of sterile Listeria Selective Enrichment Broth (Oxoid) was added and homogenized with a stomacher and incubated at 30°C for 48 h, the cultures were surface streaked onto Tryptic soy agar (Oxoid CMO131) and incubated at 30°C for 24-48 h. All Cheese samples were kept in cleaned poly styrene and stored at room temperature for 7 days in the same sales conditions. Above tests were carried out after 2, 4 and 7 days of cheese manufacture, except detection of Salmonella and Listeria were carried out after 2 and 7 days.

2.3. Chemical Analyses

Moisture and NaCl contents were determined in cheese according to AOAC. 14^{th}.16, 260 (1984) and AOAC. 14^{th}.16, 272 (1984), respectively. The pH values (Inolab 720) in cheese were measured according to the 14022 AOAC (1975) method. Fat and Nitrogen content were determined in cheese according to AOAC. 14^{th}.16, 284 (1984) and AOAC. 14^{th}.16, 284 (1984), respectively.

3. Results

The changes of different microbial groups investigated during the storage of local Yemeni cheese are shown in (Figure 1- Figure 5) and Table 1. All the microbiological analyses were carried after 48 hrs of cheese production.

As results in figure 1 demonstrate, during the storage, smoked and non-smoked cheese (sample A, C, D and E) the total aerobic bacteria counts increased from 8.91, 8.86, 9.4 and 9.4 Log cfu/g to 11.97, 10.1, 9.8 and 10.1 Log cfu/g, respectively, within 48 hrs. Furthermore, 4-7 days after production, the total count of bacteria was reduced to 9.04, 9.3, 9.3 and 9.79 Log cfu/g, respectively. As to sample B the total aerobic bacteria counts gradually increased to the value 9.8 Log cfu/g. In samples A, B, C, D and E the total counts of moulds and yeast gradually increased to 6.6, 7.6, 5.6, 8.9 and 6.5 Log cfu/g, respectively, after 168 hrs of production (Figure 2).

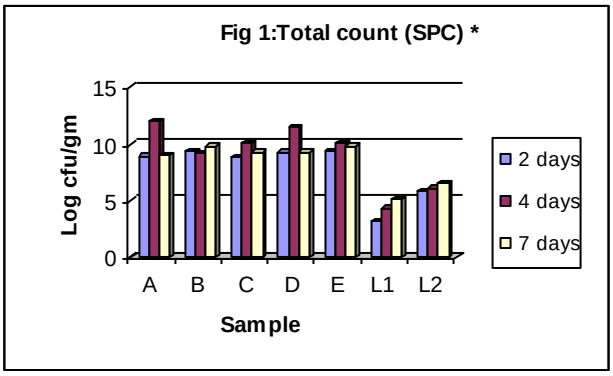
Lactic acid bacteria (LAB) count was 9.39 Log cfu/g in sample A after 48 hrs of production and then dropped to 7.1 Log cfu/g after 168 hrs. However, in samples B, C, D and E a slight increase from 9.37, 9.39, 9.3 and 9.4 Log cfu/g to 9.5, 10.36 , 9.56 and 9.9 Log cfu/g, respectively was recorded after 7 days of production (Figure 3).

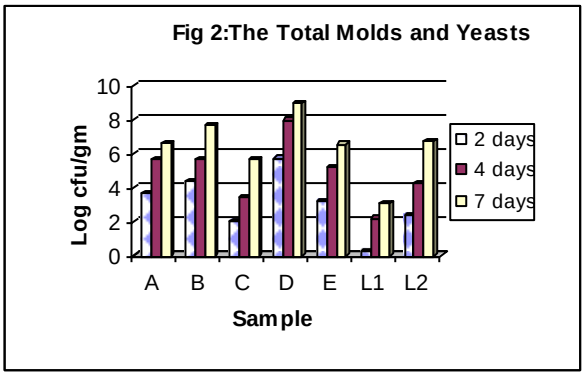
A rapid increase in the coliform count to 8.1 Log cfu/g after 7 days of production (sample A). No coliform was detected in sample D and after 2-days of production in sample C, it reached to 4.5 Log cfu/g after 4-days and dropped to 2.8 Log cfu/g at the end of storage. In samples B and E, the coliform count gradually decreased from 6.59 and 11.8 to 4.49 and 6.7 Log cfu/g after 7-days of cheese production, respectively (Figure 4).

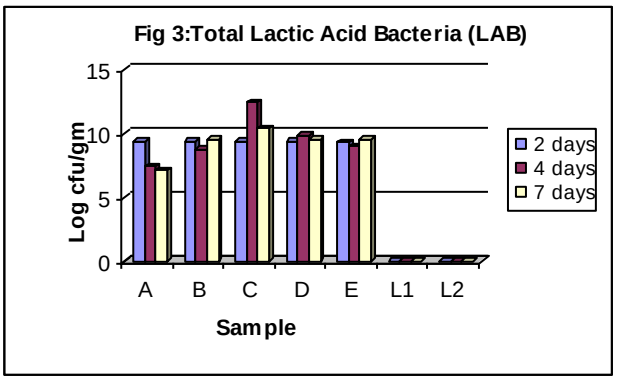
A rapid increase in the *Staphylococcus* sp. count from 3.88 to 13.4 Log cfu/g was recorded after 7-days of production (samples A). However, in samples B, C, D and E *Staphylococcus* sp. count increased from 5.7, 2.7,3.6 and 4.9 to 8.8,7.8,9 and 8.7 Log cfu/g within 2-days, then decreased to 6.88,7.4,7.1 and 6.5 Log cfu/g after 7-days, respectively (Figure 5).

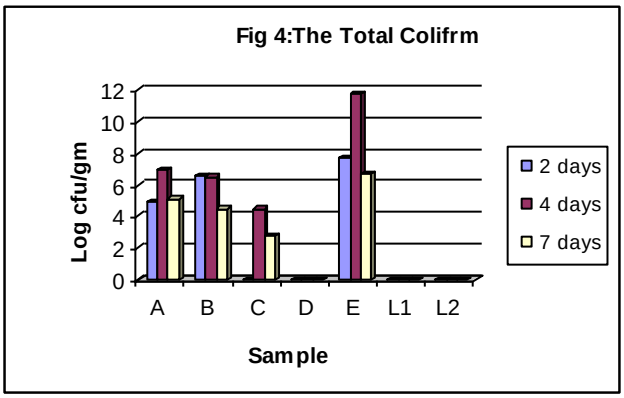
Table 1 displays the presence of *Salmonella* and *Listeria* in samples during storage period. Listeria was detected in all samples after 2 days of production except sample L1, but no *Listeria* observed in samples D and E after 7 days of cheese production. However, *Salmonella* was detected in samples A, B and E during 2-7 days of storage period but no *Salmonella* observed in samples C, D and L1.

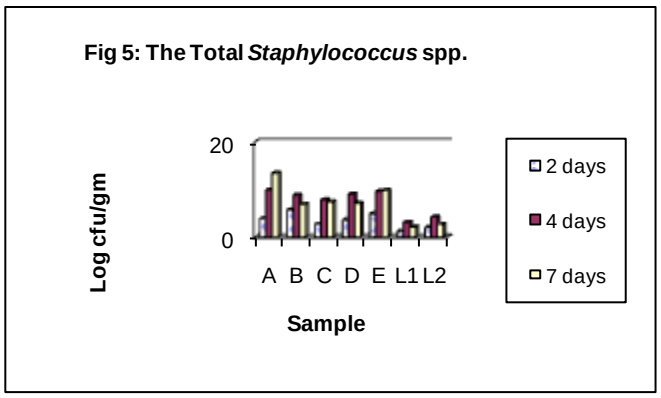
Figure 1-5. Microbiological changes in total count (Fig.1) Molds & yeasts (Fig.2) Lactic acid bacteria (Fig.3) Coliform (Fig.4) *Staphylococcus* (Fig.5) during storage of cheese samples.











* A and B: cheese samples collected from Al-bab alkabeer Market

C,D and E: cheese samples collected from Albarh market

L_1 and L_2: cheese samples prepared in the laboratory.

A,B,C,D,L1 : smoked cheese

E,L2 : non smoked cheese

Table.1 The presence of *Salmonella* and *Listeria* in samples during storage period

sample	2nd day		7th day	
	Listeria	Salmonella	Listeria	Salmonella
Smoked cheese A	+	+	+	+
Smoked cheese B	+	+	+	+
Smoked cheese C	+	-	+	-
Smoked cheese D	+	-	-	-
Non smoked cheese E	+	+	-	+
Smoked cheese L1	-	-	+	-
Non smoked cheese L2	+	-	+	+

Comparing the results of microbiological examination in figures 1-5, and table 1 with cheese samples produced in laboratory under the optimum conditions, we found that in sample L1 the total plate count, molds and yeast and *Staphylococcus* sp. started increase from 3.2, 0.3 and 1.2 Log cfu/g to 5.1, 3.1 and 2.1 Log cfu/g, respectively during 2-7 days of manufacture. No lactic acid bacteria, *Salmonella* and coliform were isolated at the end of storage period. *Listeria* was detected in sample L1 after 7-days of manufacture. In contrast, in sample L2 (non smoked cheese produced in laboratory under the optimum conditions) the total plate count, molds and yeast and *Staphylococcus* sp. started slowly increase from 5.8, 2.4 and 2 Log cfu/g to 6.5,6.7 and 2.7 Log cfu/g, respectively during 48-168 hrs of manufacture. Lactic acid bacteria and coliform were not observed.

The increase in count during the first week of storage was accompanied by a sharp drop in pH values changing from 5.4 to 4.1 (Figure 6) which is a consequence of the production of acid by the microorganisms. Chemical analysis results of smoked and non-smoked cheese samples are presented in Table 2. As shown that, the average moisture of smoked cheese samples was 47.2% changing between 44.6 and 51.73 % .However, moisture of non smoked cheese sample was 58.02%. The average of fat content of smoked cheese samples was 21.2% changing between 20.05 and 22.95% and fat content of non smoked cheese sample was 19.75%. The protein content of smoked cheese samples changed from 12.02-15.02%, the average was 13.9%. However, the protein content of non smoked cheese sample was 15.27%. The salt content of smoked cheese samples changed from 4.6-5.72%, the average was 5.2%. However, the salt content of non smoked cheese sample was 3.21 % (Table 2).

4. Discussion

Smoked cheese is a regional cheese produced in Taiz. The cheese is produced by local traditional methods to meet family needs and consumed in some regions in Yemen. The production of this cheese is seasonal and restricted to a very specific area, or because it is not possible to produce it industrially, relatively small quantities are made. There are no statistics available on production. In our study microbiological quality of cheese was not good and the total aerobic bacteria counts varied between 8.86 and 11.97 Log cfu/g. also, the total counts of moulds and yeast reached to 7.6 and 8.9 Log cfu/g in some samples (Figure 1 and Figure 2).

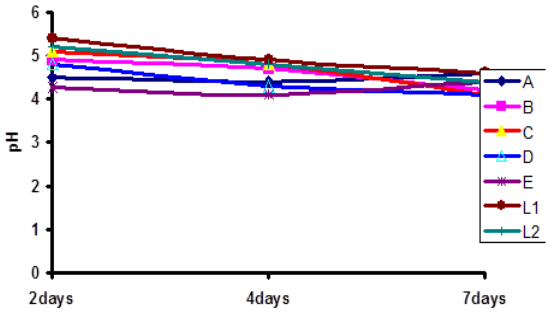


Fig: 6 Changes of pH value during storing cheese samples.

Table 2. Some chemical composition of the local Yemeni cheese samples.

sample	Moisture %	Fat %	Protein %	Salt %
Smoked cheese A	46.44	22.95	13.72	5.72
Smoked cheese B	44.6	21.8	14.9	5.02
Smoked cheese C	45.9	20.07	15.02	4.6
Smoked cheese D	51.73	20.05	12.02	5.44
Non smoked cheese E	58.02	19.75	15.27	3.21
Smoked cheese(L1)	44.09	21.6	18.7	4.3
Non smoked cheese (L2)	56.9	21.6	18.7	4.3

These results might be due to the poor sanitary conditions during cheese processing. In fact, local Yemeni cheese usually produced under traditional conditions and is handled at various stages, thus, various types of microorganism may contemned during cheese making and subsequent handling on the other hand. Total aerobics increased during storage period and a slight decrease was noted at the end of storage time. These results were similar to those reported by (Pazakova *et.al.,* 2001) in sheep cheese .

The counts of lactic acid bacteria (LAB) exhibited an increase during storage period in some cheese samples (Figure 2) and decreased in other samples. Because, LAB have been use for centuries in the fermentation of foods,

not only for flavor and texture development but also for their ability to produce antimicrobial compounds such organic acid, hydrogen peroxide and bacteriocin, which prevent the growth of spoilage and pathogenic bacteria. Similarly, in Caprino dÕAspromonte (Caridi *et al.,* 2003a) and Pecorino del Poro (Caridi *et al.,* 2003b) cheeses, coccal-shaped LAB decreased towards the end of ripening, while the lactobacilli increased. In another study performed on Kashar, the count of lactic acid bacteria was reported to decrease from 8.24 log cfu/g to 3.10 log cfu/g after 90 days of ripening (Cetunkaya and Soyutemuz, 2006). Furthermore, development of non-starter LAB throughout ripening was reported by several authors (Buffa et al., 2001; Beuvier et al., 1997; Ortigosa et al., 2001). No LAB observed in samples L1 and L2 (Figure 2) this is due to effects of heat treatment of milk and high temperature of smoking process. It can be concluded from our study that smoking did not negatively affect the growth of non-starter lactic acid bacteria during storage of smoked cheese. The results agree with those reported by Farkye (2004).

According to our results the presence and increasing of viable coliform and moulds and yeast population during storing period was higher than accepted limits in Yemen for raw cheese. These heavily contamination levels indicate that all samples of cheese may cause serious health risks. This must be due tape water in Yemen which is not hygienic enough. Cross contamination may also have occurred during processing and handling. The increasing constant of mould and yeast during storing time could be considered for the fact that yeast and mould count could metabolize lactic acid and lower pH value (Turkoglu *et. al.,* 2003). Other authors reported that highest counts being generally reached in all the microbial groups in first week of storage in other varieties of cheese (Tornadijo *et al.,* 1995; Souza *et al.,* 2003; Abdalla and Mohammed, 2010).

The level of indicator and pathogenic microorganisms including *Staphylococcus* group bacteria found in our study were higher than standard limits accepted in Yemen for raw cheese. *Staphylococcus* sp. is often found in raw milk and in the environment of the cheese plants (equipment and personal). This organism is salt-tolerant and is able to grow under a wide range of conditions; low acid production may allow *Staphylococcus* to grow and produce enterotoxins (Olerta *et al.,* 1999). Table 1 displays the presence of pathogenic bacteria including *Salmonella* sp. and *Listeria* sp. in cheese samples and. These variations may be due to the differences in production and handling conditions. The absence of these microorganisms at the end of storing time in some samples (D and E) due to the role of LAB which, which prevent the growth of the pathogenic bacteria. In samples cheese L1 and L2 the presence of *Listeria* sp. and *Salmonella* sp. may be due to cross contamination during handling or as Ramsaran *et al.* (1998) reported that the surviving pathogens may grow to high cell counts during the ripening and storage of soft cheese and this effect is more pronounced at the cheese surface, because the rapid increase in the surface pH of smear cheeses favors the growth of *Listeria* sp., which resides in ecological niches in cheese factories. The number of research studies conducted on *Listeria* sp. and *Salmonella* sp. contaminations in smoked cheeses was limited. It has been reported that Listeria and pathogenic bacteria was only recovered from 12.5% of smoked cheese samples (Al-Zoreky, 1998). Kinderlerer *et al.,* (1995) reported that the presence of *P. roqueforti*, especially the strains that possess high proteolytic and lipolytic activities, tends to inhibit the survival of pathogenic microorganisms, such as *E. coli* and *Staphylococcus* sp. Some fungal metabolites in mould-ripened cheeses were reported to contain natural *listeria* inhibitors. *G. candidum* produces two components, d-3-phenyllactic acid and d- 3-indollactic acid, which can inhibit *L. monocytogenes* (Dieuleveux *et al.,* 1998).

In our study, various factors contribute to the decline of these microorganisms during storage, they include smoking process, increase in concentration of NaCl and inhibition of these bacteria by lactic acid bacteria by causing decrease in pH. When we compared these results to the cheese samples that manufactured in laboratory under hygienic conditions we found that was best quality than locally cheese. The hygienic quality of smoked cheese was best than non smoked cheese this is due to effects of antibacterial and antioxidant effects of the smoke components such as formaldehyde, carboxylic acids, and phenols (Goulas and Kontominas, 2005).

When the results of our chemical analysis are compared with previous studies (Table 2), the moisture content is seen to be higher than that found by (Al-Zoreky, 1998) but consistent with the results of (Souza et al., 2003) in Serrano cheese. The fat and protein percentage were similar to (Mirzaei *et al.,* 2008) and (Turkoglu *et al.,* 2003) in other varieties of cheese but lower than the findings of researchers (Kamber and Celik, 2007; Kocak *et al.,* 1996 ; Arici and Simsek, 1991). The salt contents in cheese samples were similar to that found by Cetunkaya and Soyutemuz (2006), Kamber and Celik (2007) and higher than the findings of other researchers (Mirzaei *et al.,* 2008; Souza *et al.,* 2003). It is important to point out that the salt content of the different cheeses is fairly irregular due to the salting technique used. Because this non-standard production style is excessive the compositions and quality of cheese vary depending on the experiences and working conditions of the masters performing the production ,in addition to the types of milk (raw or reconstituted) which used in manufacture of cheese.

5. Conclusion

With this research it is determined that smoked cheese offered to the market for consumption in Yemen was low

quality and contaminated with pathogen. This contamination may cause important public health risks. We concluded that standardization of the smoked cheese production, the use of high quality raw materials, production in modern enterprises and hygienic conditions will be effective in prevention of the probable dangers in terms of public health.

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Animal Trade in Amman Local Market, Jordan

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Abstract

The magnitude of animal trade in Amman city, Jordan, was evaluated during July to November 2009. Birds have constituted the majority of specimens in trade with the sum quantity of 16942 specimens, represented by 54 species among 19 families. In addition, reptiles were also encountered, with a total of three species belonging to three families. Mammals were the least represented group with four species belonging to three families and a total of only nine specimens in trade**.** Twenty-three species of the traded birds were included in CITES appendices, where 16 and 7 species are listed in appendix II and III, respectively. Only one species of reptiles is under CITES lists, while none of the traded mammals are included under any CITES category**.**

Keywords: Animal trade, Jordan, CITES, birds, mammals, reptiles**.**

1. Introduction

Jordan, a Middle Eastern country with an area of 89,342Km² and a population of 5.97 million estimated in 2009. Despite that desert constitutes about 75% of the total size area of Jordan; it enjoys various habitats ranging from the Mediterranean forests, black lava, gravel deserts, and arid sandstone mountains. The heterogeneous habitats present in Jordan allow the presence of an enormous biodiversity contents, where a total of 78 mammalian species (Amr, 2000), 107 reptiles and amphibians (Modry *et al.*, 1999; Disi *et al*., 2001; Abu Baker *et al*., 2005) and 425 bird species (Andrews, 1995) and around 2500 plant species (Al Eisawi, 1996) were recorded.

Jordan is considered a leading country in the Middle East in biodiversity conservation, where eighteen acts and eight regulations include provisions on environmental protection are present. These laws and regulations are enforced through different governmental and non-governmental agencies. As far as nature conservation and wildlife protection goes, Agricultural Law No. 44 of 2002, Aqaba Region Authority law No. 32 of 2000 and the Law of Environmental Protection No. 1 of 2003 are in effect. Articles in these laws include protection of birds and wild animals and their hunting regulations, designate cooperation between different governmental agencies with competent authorities, assign responsibilities and power to act-enforcing the different articles, and distribution of the financial resources and available funds among agencies (Amr *et al*., 2004).

In addition to national laws, Jordan pays attention to the role of international agreements for the protection of wildlife from overexploitation. Accordingly, it gets into force within the provisions of the Convention in International Trade in Endangered Species of Wild Animal and Plant (CITES), four years after the enforcement of this convention, and considered party number 47 in the chronological order of the world countries that have signed CITES convention, as well as party number four in the Middle East.

Since the Royal Society for the Conservation of Nature (RSCN) is the only NGO in the Middle Eastern countries with a mandate from the government of Jordan to establish, manage and operate nature reserves in Jordan, besides taking the full responsibilities for managing wildlife protection, hunting monitoring and control. It acquired a formal delegation from the Ministry of Agriculture (MOA) to acts side by side with the veterinarian department of the MOA, as the management authority of the CITES convention. Accordingly, the RSCN has been active on CITES issues in both global and regional contexts and has acted to the best of its ability to minimize activities and impacts determinant to Jordanian biodiversity.

Jordan is considered a passage for smuggling of animals to countries in the Arabian Peninsula and elsewhere in the Middle East. Shipments of reptiles (snakes and tortoises) were confiscated at the Jordanian border with Syria to be smuggled to Saudi Arabia (Amr *et al.*, 2007). Similarly, birds and mammals were confiscated at the Iraqi borders with Jordan (RSCN, personal communications).

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Regionally, very little was mentioned on trade of animals and plant species. Amr *et al.*, (2007) described the animal trade in Syria, and addressed the illegal trade of reptiles in Damascus Animal Market. Moreover, Soorae *et al.* (2008) conducted a survey on the trade in wildlife as pets in the United Arab Emirates. Further notes on the illegal wild animals trade was described from Lebanon by Dakdouk (2009).

In Jordan, virtually no information was reported concerning animal trades. However, it is practiced in two forms, animal pet shops that are licensed and subjected to a routine check- up by the RSCN, and in streets, especially on Fridays, the official day-off in Jordan, where venders, hobbyists and hunters sell their animals in cages; this practice has been ongoing for many years, and, occasionally, the RSCN staff confiscates illegal items.

This study is the first of its kind from Jordan and it aims at identifying the magnitude of illegal animal in trade at the Local Market in Amman, Jordan, in terms of species that are in trade, their volumes, and number of CITES species present in the market.

2. Materials and Methods

A Total of 10 visits to Local Market were carried out between July and November 2009. These visits were conducted by a group (three - four persons) of researchers from the RSCN and Birdlife International who inspected the animals that were in trade in Local Market in Amman city. Visits involved an early inspection of the market, in order to identify all species present to produce a species list. In addition, number of individuals of each species was counted and their prices were obtained. Origins of these animals were obtained when applicable.

Figure 1. Sun Conure and Timneh Gray Parrot offered for sale in Amman Local Market. Both species are listed under CITES appendix II.

3. Results

Ten visits were undertaken to evaluate the magnitude of animal trade in Amman Local Market. Three major groups of vertebrates were found in the Local Market in Amman. Birds have constituted the majority of specimens in trade with sum quantity of 16942 specimens, represented by 54 species among 19 families (Table 1, Figure 1-2). In addition, reptiles were also encountered, with a total of three species belonging to three families, and a volume of 86 specimens. Mammals were the least represented group with four species belonging to three families and a total of only nine specimens in trade in the Local market (Table 2).

Figure 2. Caged Senegal Parrots "CITES species" for sale in Amman Local Market.

Table 1. Bird species in trade in Amman Local Market, NC is not listed.

Common Name	Scientific Name	CITES Status	Total No. in Trade	Price / bird in US$	Total price in US$
	Family: Numididae				
Helmeted Guineafowl	*Numida meleagris*	NC	2	21.2	42.4
	Family: Phasianidae				
Chukar	*Alectoris chukar*	NC	58	33.9	1968.3
Pheasant	*Phasianus colchicus*	NC	51	38.9	1983.2
Sand Partridge	*Ammoperdix heyi*	NC	4	49.5	198
Indian Peafowl	*Pavo cristatus*	NC	7	88.4	618.6
Black Francolin	*Francolinus francolinus*	NC	1	21.2	21.2
	Family: Falconidae				
Lesser Kestrel	*Falco naumanni*	II	2	28.3	56.6
Kestrel	*Falco tinnunculus*	II	25	31.1	839.9
	Family: Accipitridae				
Long- legged Buzzard	*Buteo rufinus*	II	3	70.7	212.1
	Family: Rallidae				
Moorhen	*Gallinula chloropus*	NC	1	2.8	2.8
	Family: Columbidae				
Ring Dove	*Streptopelia risoria*	NC	94	21.2	1993. 8
Diamond Dove	*Geopelia cuneata*	NC	3	42.4	127.2
Palm Dove	*Streptopelia senegalensis*	III	1	3.5	3.5
Turtle Dove	*Streptopelia orientalis*	NC	3	3.5	10.6
	Family: Psittacidae				
Budgerigar	*Melopsittacus undulates*	NC	1527	12.6	19173.9
Fisher's lovebird	*Agapornis fischeri*	II	59	46	2711
Peach-faced Lovebird	*Agapornis roseicollis*	II	84	42.4	339.4
Nyasa Lovebird	*Agapornis lilianae*	II	48	46	92

Congo African Grey Parrot	*Psittacus erithacus*	II	84	282.8	23755.7
Timneh African Grey Parrot	*Psittacus erithacus timneh*	II	48	120.2	5769.2
Alexandrine parakeet	*Psittacula eupatria*	II	13	494.9	6433.8
Ducorp's cockatoo	*Cacatua ducorpsii*	II	2	424.2	848.4
Senegal Parrot	*Poicephalus senegalus*	II	34	70.7	2403.9
Cockatiel	*Nymphicus hollandicus*	NC	155	74.2	11506.6
Sun Conure	*Aratinga solstitialis*	II	10	131.5	1315
Blue-fronted Amazon	*Amazona aestiva*	II	13	353.5	4595.6
Ring-necked Parakeet	*Psittacula krameri*	III	26	56.6	1470.6
Eastern Rosella	*Platycercus eximius*	II	2	106	211.9
	Family: Strigidae				
Little Owl	*Athene noctua*	II	1	14.1	14.1
	Family: Upupidae				
Hoopoe	*Upupa epops*	NC	7	17.7	123.7
	Family: Paridae				
Great Tit	*Parus major*	NC	6	7.1	42.4
	Family: Alaudidae				
Crested Lark	*Galerida cristata*	NC	4	1.4	5.7
Temminck's Lark	*Eremophila bilopha*	NC	25	3.5	88.4
	Family: Pycnonotidae				
Yellow-vented Bulbul	*Pycnonotus xanthopygos*	NC	302	2.8	854.1
White-eared Bulbul	*Pycnonotus leucotis*	NC	39	17	661.8
	Family: Nectariniidae				
Palestine Sunbird	*Nectarinia osea*	NC	50	22.3	1117.1
	Family: Passeridae				
Rock Sparrow	*Petronia petronia*	NC	22	2.1	46.7
House Sparrow	*Passer domesticus*	NC	3	1.4	4.2
	Family: Ploceidae				
Golden Bishop	*Euplectes afer*	III	2	21.2	42.4
	Family: Estrildidae				
Zebra Finch	*Taeniopygia guttata*	NC	2874	6.4	18287.6
Indian Silverbill	*Lonchura malabarica*	NC	806	4.2	3419.1
Lavender Waxbill	*Estrilda caerulescens*	III	10	10.6	106.1
Cut-throat Finch	*Amadina fasciata*	III	23	8.5	195.1
Java Finch	*Padda oryzivora*	II	11	21.2	233.3
	Family: Viduidae				
Long-tailed Paradise	*Vidua interjecta*	III	61	31.8	1939.2
Pin-tailed Whydah	*Vidua macroura*	III	2	24.7	49.5
	Family: Motacillidae				
Yellow Wagtail	*Motacilla Flava*	NC	1	28.3	28.3
	Family: Fringillidae				
Greenfinch	*Carduelis chloris*	NC	325	1.4	459.6
Goldfinch	*Carduelis carduelis*	NC	1221	42.4	51795.8
Linnet	*Carduelis cannabina*	NC	123	1.4	173.9
Desert Finch	*Rhodospiza obsoleta*	NC	266	2.8	752.3
Canary	*Serinus canaria*	NC	8317	21.2	176406.9
Sinai Rose finch	*Carpodacus synoicus*	NC	76	3.5	268.7
Siskin	*Carduelis Spinus*	NC	5	2.8	14.2
	Total		**16942**	**3040.1**	**345835.4**

Twenty-three species of the traded birds were included in CITES appendices, where 16 and 7 species are listed in appendix II and III, respectively. Only one species of reptiles is under CITES lists, while none of the traded mammals are included under any CITES category (Table 2, Figure 3).

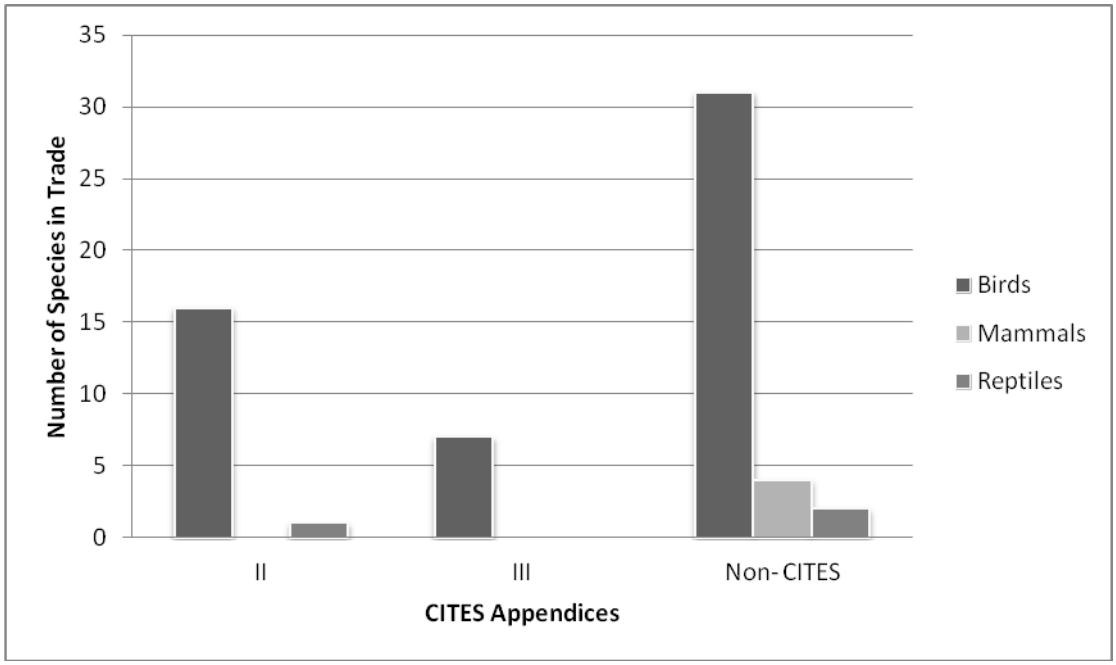

Figure 3. Number of species according to their CITES status

Table 2. Summary for CITES and non-CITES animal trade in Local Market

	No. of species	CITES		Non-CITES	
		No.	%	No.	%
Birds	54	23	43.4	30	56.6
Mammals	4	0	0	4	100
Reptiles	3	1	33.3	2	66.7

A total of 439 specimens of CITES- II species were in trade in the public market. The majority (90.4%) of these specimens belong to the Family Psittacidae. Among these, the Peach-faced Lovebird and the Congo African Gray Parrot were the most (19.1%) species in trade. Meanwhile, family Strigidae represented by the Little Owl was the least (0.2%) species in trade.

Also, a total of 125 specimens of CITES- III species were in trade, including 63 specimens of Family Viduidae, which constituted the majority of specimens in trade and among it, the Long-tailed Paradise was the major (48.8%) bird in trade (Figure 4). However, family Columbidae represented by the Palm Dove was the least (0.8%) bird in trade.

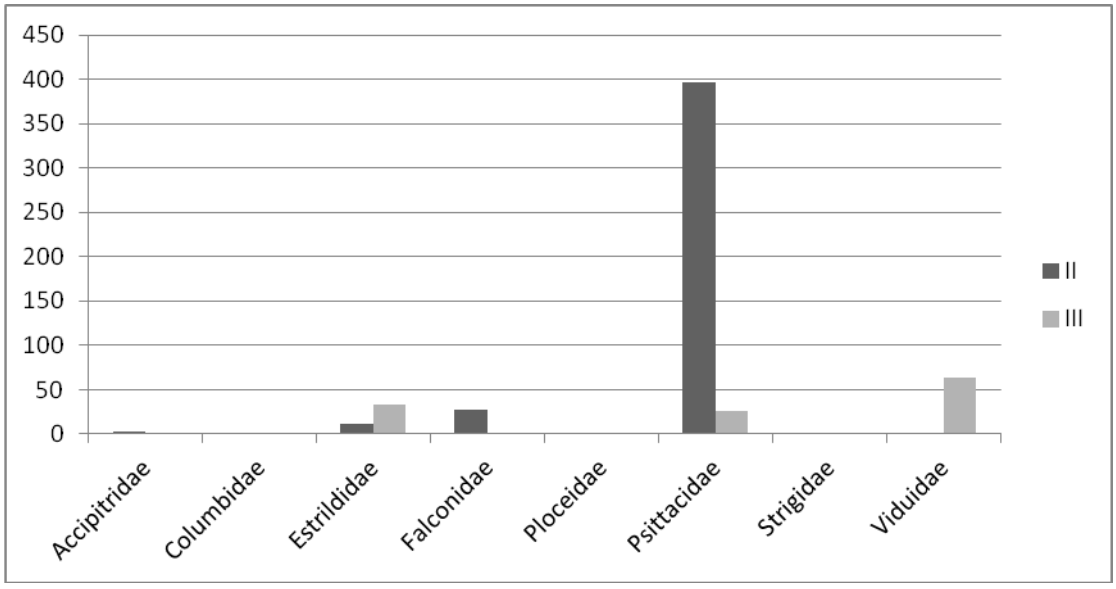

Figure 4 . Highest traded families according to their CITES status

Bird prices ranged from as low as 1.5 US$ for the Hoopoe, White-eared Bulbul and others to as high as 525 and 450 US$ for the Alexandrine parakeet and Ducorp's cockatoo, respectively (Table 1).

Local and cage-bred birds were the most (96.7%) common species in trade and accounted for 16378 specimens. Local birds were either captured from Jordan or Syria and included the Kestrel, Long- legged Buzzard, Temminck's Lark and others. Pheasant, Chukar, and the Ring Dove are bred in captivity for trade. Ring-necked Parakeet and the Indian Silverbill are introduced species through cage escapes that now breed at several areas in the wild.

The Budgerigar, Goldfinch, Canary and the Zebra Finsh were the most species encountered accounting for 82.3%. These birds are imported legally from various countries and some are locally cage-bred.

Other local birds and some of the migrant bird species in trade were taken directly from the wild, either trapped from the mountains of north Jordan or the Jordan Valley as free-flying adults or taken as nestlings.

All four mammalian species in trade are of local origin. None of the mammals species are under CITES. The number of observed animals is very limited (Table 3). These animals are either trapped or picked up from the forests (Sciurus anomalus and Eiranicius concolar) or from the desert (Allactaga euphratica).

Table 3. Mammal species in trade in Amman Local Market.

Common name	Scientific name	CITES status	Total No. in trade
Long- eared Hedgehog	*Hemiechinus auritus*	NC	1
East European Hedgehog	*Eiranicius concolar*	NC	1
Persian Squirrel	*Sciurus anomalus*	NC	5
Five- toed Jerboa	*Allactaga euphratica*	NC	2

Three species of reptiles are in trade. Reptiles are either local (*Testudo graeca* and *Natrix tessellata*) or exotic (*Trachemys scripta*). Only the Mediterranean Spur- thighed Tortoise is listed under appendix II. These animals are also collected from their original habitats in Jordan

Table 4. Reptilian species in trade in Amman Local Market.

Common name	Scientific name	CITES status	Total No. in trade
Mediterranean Spur-thighed Tortoise	*Testudo graeca*	II	42
Dice Snake	*Natrix tessellata*	NC	15
The Red-eared slider	*Trachemys scripta*	NC	29

4. Discussion

Despite all the effort by the RSCN to organize and minimize the illegal animal trade in Jordan, the large number and available of illegally sourced species in the market highlighted the need for further enforcement efforts on the part of the relevant authorities. The scale of animal trade in the Local Market in Amman reaches about 115,000 US$ per month. This is comparably high taken into consideration the low annual income per capita (about 3000 US$). Prices for animals are variable compared to those reported in the pet trade shops in the United Arab Emirates (UAE), for example, the Senegal Parrot is offered for 70 US$ in the Amman Local Market, while offered for sale at a price of 325 US$ in the UAE, while the Alexandrine parakeet is offered for about 495 US$ in Amman compared to a maximum of 210 US$ in UAE (Soorae *et al.,* 2008). Such variations in prices are attributed to the origin of the specimen, its general health and demand. Moreover, it was apparently noticed that CITES- II species are more expensive than CITES- III and the unlisted species which is in accordance with Courchamp *et al.* (2006) who stated that CITES listed species are more expensive than non- CITES species.

Contrary to other countries in Europe (Spellerberg, 1976; Auliya, 2003), demand on reptiles and mammals is very limited, whereas most of the demand is focused on birds. All mammals and reptiles are of local origin with the exception of the Red-eared Slider. Only four mammalian and two reptilians were found in trade. On the other hand, illegal trade in reptiles in Syria is more than that of Jordan, where at least five species were found in trade in very high number of individuals (Amr *et al*., 2007).

The number of bird species traded in Amman Local Market and listed under appendix II and III were 16 and 7 respectively, compared to 6 and 20 species in UAE listed under appendix I and II respectively (Soorae *et al.,* 2008). No bird species listed under appendix I were found in trade in the Local Market, this is due to the extremely high prices of such animals that are not affordable by the local people who attend the Local Market.

As shown in table 1, most birds in trade are either of local origin (Sand Partridge, Turtle Dove, Palm Dove etc.) or breed in captivity (Pheasant, Canary etc.). Some local birds are trapped at a large scale (the Greenfinch, Goldfinch and the Linnet) and may impose a threat to their local status. Such illegal trade will certainly affects these species and will lead to a drastic decline in their populations. For example in Morocco, Lambert (1979) suggested that the net effect of collecting *T. graeca graeca* may have reduced pre-trade population levels by as much as 86%.

Dakdouk (2009) stated that an export ban for *T. graeca* was established in 2004 since trade in this species is popular in the pet trade in Lebanon and there is a high number of wild and captive specimens exported which raise concerns in Lebanon.

Trade of *Natrix tessellata* is not a common practice in Jordan and it is rarely sold in pet shops, as it is not a popular snake for husbandry due to its nervous behavior and foul odor. It is neither consumed by locals nor prescribed as a source of folk medicine (Amr *et al.,* in press).

Overharvesting of certain species addresses the need to evaluate the level of trade and make sure that it is not causing declines in wild populations. In Jordan, no records to track the imports and exports of reptiles are available. The lack of information implies that population declines due to overexploitations could be going undetected (Schaepfer *et al.,* 2005). Further investigation should focus on the actual number of traded animals in Jordan.

In order for CITES to be an effective conservation tool, it is imperative that parties recognize the scale of international trade in birds species as they constitute the majority of traded specimens in the market. And thus, a response should be performed accordingly especially at the entrance points of Syria by enhancing the enforcement and awareness to CITES convention.

5. Conclusions

Animal trade in Amman Local Market reflects the enforcement of CITES in Jordan. About 60% of species in trade are not listed under CITES appendixes, however, only 16 and 7 species are listed under appendix II and III respectively. Birds were the most traded animals since they are favored by local people. Further studies are urgently required to evaluate the animal trade in Jordan in licensed pet shops in Amman and major cities. In addition, public awareness, law enforcement and routine inspection should be implemented on a larger scale to minimize trade in CITES animals in Jordan.

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Short Communication

A modified Smoking Machine for Monitoring the Effect of Tobacco Smoke on Albino Rats

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Abstract

We describe a modified smoking machine to be used for monitoring the effects of narghile and cigarette tobacco smoke on experimental animals; a vacuum pump, a time controller, and an electronic valve that control the sequence of puff- and fresh air-inlet and exit into and out of the inhalation chamber. The design allows intake of enough tobacco smoke and prevents oxygen deprivation in the inhalation chamber.

Keywords: Smoking machine, tobacco smoke, albino rats.

1. Introduction

Tobacco smoking is practiced nowadays by over one billion people, and is deemed responsible for about five million premature deaths per year worldwide; it stands behind or is related to many health problems, which makes it a serious risk to human health (Wolfram *et al.,* 2003; Al-Safi *et al.*, 2009; Neergaard *et al.,* 2007). There are different methods of smoke tobacco, notably cigarettes, cigars, pipes, and water-pipes (narghile).

The smoke of burning tobacco is divided into mainstream and sidestream smoke. The mainstream smoke emerges from the tobacco product through mouth during puffing, whereas sidestream smoke comes from the burning cone and from the mouth during puff intermission (Hoffman and Wynder,1986). When a cigarette is smoked, the atmosphere becomes contaminated with both mainstream and sidestream smoke. Sidestream comprises about 95% of the cigarette smoke air contamination (Shephard, 1982).

The combustion of a typical cigarette involves perhaps 102-second puffs and 550 seconds of sidestream combustion; about 46% of the tobacco is burnt during the puff phase (Hoegg, 1972).

Many smoking machines have been constructed, mainly for studying chemical the composition of cigarette tobacco smoke and its effect on the human body (Baeza-Squiban *et al.,* 1999; Baker *et al*., 2004; Chen *et al.,* 2008; Counts *et al*., 2005).

Rats, mice, and guinea pigs are used as experimental mammalian animals to study the effect of smoke on the structure and function of different organ systems of tested animals. Liswi (1988) exposed albino rats to the smoke of 3 different types of Jordanian made cigarettes for 3 months. She used a simple inhalation chamber that was connected to vacuum pump, with continuous smoke flow. The inhalation time lasted between 5-6 minutes to finish the burning of the cigarettes. AL-Kurd *et al*. (2002) developed a special smoking machine to study the effect cigarette mainstream on guinea pigs. Their system of timing of the puff duration gave one (2.5 seconds)/minute. The smoking period was 3.5 and 5.5 months. The effect of smoking on the histology of animals was not obvious. There is a need for increased puff duration in hope for getting histological effects in shorter exposure time (3 months). But at the same time, it is important to supply the animals with fresh air to prevent anoxia.

2. Design of the Smoking Machine

An automated smoking machine was designed with a special smoking topography, suitable for the exposure of rats to narghile-tobacco smoke. The machine is composed of the following components:

1. Narghile/cigarettes.
2. An inhalation chamber made of plexiglass (8mm thick), with the following dimensions (30cm length x 22.5cm width x 10.5cm height) that can host up to five rats.
   Larger chambers can be made for larger animals.
3. A vacuum pump (Vacuubrand MZ 2, Germany).
4. An electronic valve made at the Electrical Workshop/ Department of Physics/ University of Jordan).
5. A time controller, made at the Electrical Workshop/ Department of Physics/ University of Jordan. It controls the sequence of operation of the pump and the valve.
6. 30% and 50% alcohol traps connected in series by rubber and glass connectors.

The whole system is illustrated in Figures 1-2.

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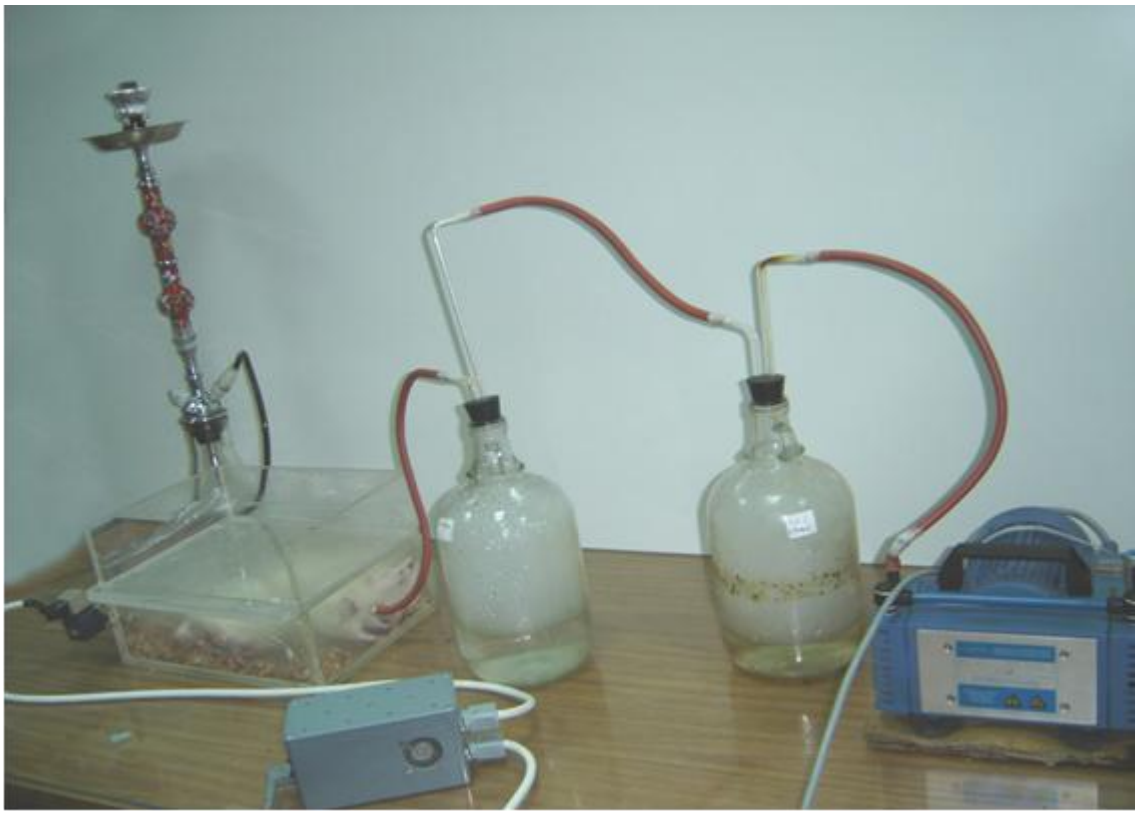

Figure1. Set-up of the automated smoking machine, used to expose the rats to narghile smoke.

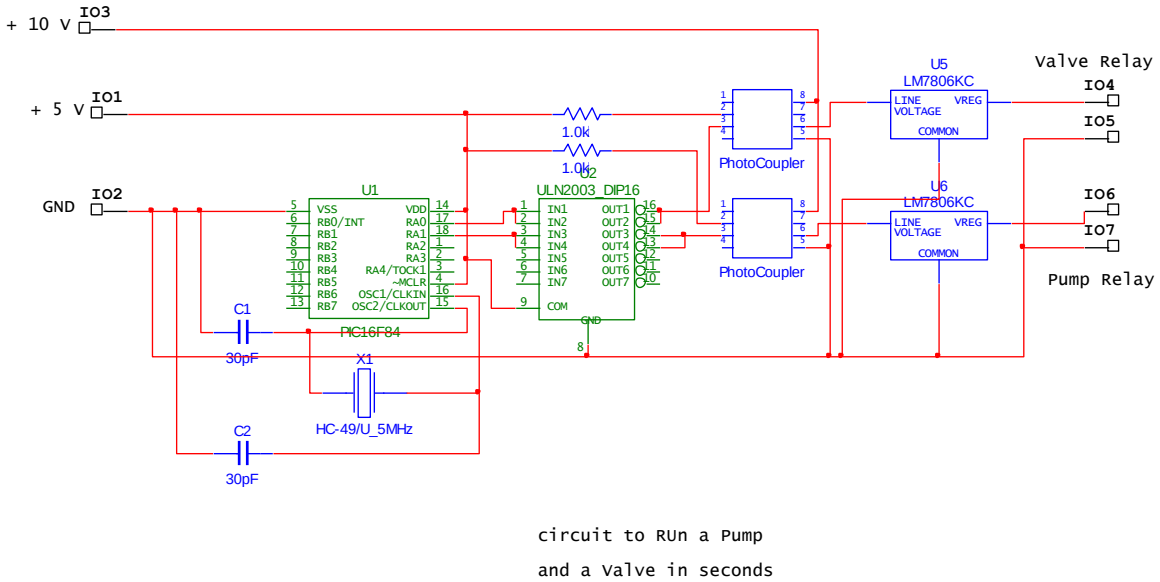

Figure 2. A circuit to run a pump and a valve in a digital smoking machine.

2.1. *The smoking regimen*

Each cycle of the smoking regimen lasts for 90 seconds and consists of three successive steps, operating as follows:

1. Narghile (cigarette) smoke is drawn through the inhalation chamber continuously for 30 sec.
2. An inlet to fresh air is then opened, allowing fresh air to be introduced instead of smoke, which will be washed out of the chamber. The washing out process will also take 30 sec.
3. In the last 30 sec, the vacuum pump will be turned off, and rats will be allowed to breathe fresh air normally.

3. Results and Discussion of the Smoking Machine

Two important points have to be stated concerning the smoking machine:

1. The design took into consideration the prevention of oxygen deprivation and poisoning by toxic gases such as CO poisoning in the inhalation chamber. This has been avoided via splitting the smoking cycle into three equal periods involving: introducing narghile

(cigarette) smoke, washing it out with fresh air, and, finally, letting the rats to breathe smokeless air normally. The 30 sec duration was chosen in accordance with the fact that narghile tobacco does not have the self-burning characteristic of a cigarette, which implies longer periods of smoke drawing, as will as with technical issues related to the time controller. Nevertheless, spacing the three parts of the smoking cycle by 30 sec, was found to be practically applicable and appropriate to readily give smoke, at the beginning of each smoking cycle.

2. Using thermometer, the temperature inside the inhalation chamber was kept at 25 °C during the smoking session. This is largely due to the aging phenomenon of narghile smoke, which results from the long distance which might be 25 times of a cigarette-covered by the narghile smoke, from the production site (the bowl), down the vertical stem, and finally its introduction into a long suction hose, preceded by bubbling through water (Chaouachi, 2009).

4. Conclusion

The smoking machine we described can be applicable for studying the effects of tobacco smoke of cigarettes, narghile and cigar on different types of experimental animals.

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Short Communication

Microbiological Quality and in Use Preservative Capacity of Shampoo Preparations Manufactured in Jordan

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Abstract

The microbiological quality of 16 different shampoo formulations (manufactured by 16 different factories) and marketed in Jordan was investigated to determine the preservative capacity of these products at the time of sale and after use. Procedures used were according to those described in ISO technical standards. Thirteen (81.25 %) of the formulations studied were found to be free of contamination. One product harbored *Escherichia coli* and 2 contained *Pseudomonas aeruginosa*. Total microbial count in the contaminated brands was $>10^5$ CFU / ml. When the contamination free products were returned after a period of normal use, 7 (53.85 %) harbored variety of gram negative and positive bacteria; high numbers of bacteria were detected in 5 (38.5 %) of the returned products. Cocamide diethanolamide in mineral salts medium supported the growth of *P. aeruginosa* ATCC 9027, whereas sodium lauryl ether sulphate was not inhibitory at the concentration usually used in shampoo formulations. It is concluded that unless adequately preserved, shampoo ingredients can support the growth of microorganisms known to cause spoilage and / or possible health problems. Manufacturers of shampoos in Jordan need to improve the in use preservation efficacy of their products before gaining the confidence of consumers.

Keywords: Shampoo, Cosmetics, Contamination, Microorganisms, Quality.

1. Introduction

Shampoo preparations are personal care products. The bulk ingredient in these formulations is water, typically making about 70 – 80% of the entire formula. The second major constituent is the primary surfactant, followed by the foam boaster. Other ingredients such as thickeners, conditioning agents, modifiers and special additives are incorporated to provide the product with additional required properties (Hőssel *et al.,* 2000).

However, the sterility of shampoos is not necessary; they must not be contaminated with pathogenic microorganisms and they should not harbor microbial contaminants in high numbers (Ravita *et al.,* 2009). It has long been demonstrated (Olson, 1967) that proliferation of *Pseudomonas* species in a shampoo preparation based on sodium lauryl sulphate as a surfactant resulted in product separation and discoloration. The rate of microbial contamination in shampoo brands marketed in a developing country was found to be 43% (Abdelaziz *et al.,* 1989). These authors found that bacterial count in the investigated products was low and pathogens were absent. Of special concern to cosmetic industries is the detection of *Pseudomonas aeruginosa* in their products. This bacterium is an opportunistic pathogen with spoilage potentials and was the most common microorganism associated with recall of cosmetic formulations in the United States and Europe (Wong *et al.,* 2000; Lundov and Zachariae, 2008)

Assessment of preservative efficacy in cosmetics is usually performed using the challenge test (ISO/WD 11930: 2008). This test provides assurance regarding the microbiological quality of the product at the time the test is performed. Russell (2003) suggested that challenge test should be undertaken at the beginning, during and at the end of the shelf life of the product.

Jordan institution for standards and metrology adopted a hair shampoo specification (JS 483: 2002) which has become mandatory since January 2003. This specification stipulates that total bacterial count should be $< 10^2$ / ml of shampoo and gram negative bacteria as well as *Staphylococcus aureus* should be absent. Unfortunately, there is no published work from Jordan that deals with this subject; the objective of this paper is to investigate the microbiological quality of shampoo brands manufactured and marketed in Jordan prior and during normal use by consumers. The ability of *P. aeruginosa* to grow at the expense of sodium lauryl ether sulphate and cocamide diethanolamide, which are the major two surfactants used in shampoo formulations was also studied.

2. Materials and Methods

A total of 16 different shampoo brands were purchased from the local market with preference to preparations

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manufactured by Jordanian companies. Each brand was manufactured by a company and was given a code in the laboratory before the experimental work was commenced. Viable bacterial count was performed for each product using the pour plate technique. Aliquot of 1.0 ml of the respective preparation was aseptically transferred to a sterile petridish and 20 ml of molten trypic soya agar supplemented with 1% tween 80 as a neutralizing agent. The plate was shaken, allowed to solidify and then incubated at 35 ºC for 48 hours before developed colonies were counted visually. When initial colony count was high, 10 fold serial dilutions were made in sterile saline and pour plate was repeated. Developed colonies were purified and identified according to the diagnostic tables given by Barrow and Feltham (1993). The entire identification tests employed were all traditional based on gram reaction, microscopy, biochemical reactions and ability to grow on certain substrates. Yeast and moulds were recovered by directly plating a loop-full of the preparation onto the surface of a dried Sabauraud Dextrose Agar, which were then incubated at room temperature (approximate to 22- 25 ºC) and inspected daily for the presence of growth before being discarded after 7 days. Isolation of *E.coli*, *P.aeruginosa* and *Staphylococcus aureus* was performed as described in ISO technical publications (22717: 2006, 22718: 2006 and 21150: 2006, respectively).

To determine the in-use efficacy of the preservative system in the shampoo brands that were found to be free of microbial contaminants, formulations were given to volunteers for normal use and then returned when the residual quantity in the container was approximate to 1/4 of its original volume (no time limit for use was given to volunteers as the container volume varied from one brand to another; but in general they were all returned in less than a month). Microbial count in the remaining aliquots of the used shampoo and the identity of the bacterial isolates in addition to the detection of specific microorganisms were all performed as given above.

The ability of *P. aeruginosa* ATCC 9027 to survive and grow at the expense of sodium lauryl ether sulphate (SLES), which is a major surfactant, used in shampoo formulations and cocamide diethanolamide (CDEA), which is a foam boaster, was established using mineral salts medium supplemented with either of the compounds as a main source of carbon and energy. Increase or decrease in number of the inoculated bacterial strain employed in this experiment was plotted in a growth curve and the assimilability of the material under investigation by the test organism was extrapolated. A cell suspension of *P. aeruginosa* ATCC 9027 was prepared by growing the bacterium for 24 hours on a plate of nutrient agar; colonies were harvested and then suspended in phosphate buffer to contain 10^7 CFU / ml. Aliquot of 1 ml of this suspension was used to inoculated 100 ml of sterile Bushnell - Has broth medium (this is a mineral salts medium devoid of any carbon source) in 500 ml flasks supplemented with either 14.5 gram of commercial SLES or 4 g of CDEA. Flasks were incubated in a shaking water bath and at intervals 1 ml aliquots were aseptically withdrawn for total viable bacterial count as given above. A flask containing Bushnell - Has broth without any carbon source was also inoculated with the same bacterial suspension to serve as a control. This was incubated and its content sampled as the other 2 flasks. All media used throughout this work were purchased from Difco -Michigan-USA

3. Results

Thirteen out of 16 shampoo preparations manufactured and marketed in Jordan were found to be free of microbial contamination. Selected organisms, namely *Pseudomanas* species, *E. coli, S. aureus , Candida* species and moulds were not detected. The remaining 3 brands were found to be heavily contaminated; total bacterial count in the 3 products exceeded 10^5 CFU / ml. One product harbored *E. coli* while the other 2 were contaminated with *P. aeruginosa*.

The formulations which were found of acceptable quality were given to volunteers for normal use. When approximately 3/4 of the bottle content was used, they were returned for further processing. Seven out of 13 returned products harbored microorganisms to various levels, varying from 70 to $l0^5$ CFU/ ml. Table 1 demonstrates types and numbers of bacteria recovered from shampoo products after being used. In brief, 2 products sustained count < 100 CFU / ml whereas, the other 5 contained high numbers (> 10^2 CFU / ml). *Bacillus* species was the most dominant bacteria; whereas each of the following bacteria; *Pseudomonas* sp., coagulase negative Staphylococci and *Enterobacter* sp. were recovered from 3 products. *Serratia* sp was detected in 2 of the returned products. However, *E. coli, Staphylococcus aureus, Candida* and moulds were not isolated.

Figure 1 demonstrates the growth curves of *P. aeruginosa* ATCC 9027 in Bushnell-Has medium with and without SLES or CDEA. It is evident from this figure that commercial grade of SLES cannot be considered as inhibitory to the test organism at the concentration used (14.5 g %). On the other hand, CDEA was definitely nutritional to the test organism at the concentration employed in the experiment.

4. Discussion

This investigation has demonstrated that 18.75 % of the studied shampoo products were heavily contaminated with bacteria (> 10^5 CFU/ ml). Contaminants included *P. aeruginosa* and *E. coli*. The former organism is an opportunistic pathogen with spoilage potential and the later is an indicator of fecal pollution. According to the Jordanian standards (JS 483: 2002) shampoo formulations should not contain more than 10^2 CFU / ml and *P. aeruginosa* as well as *E. coli* must be absent. Therefore these brands were out of specifications and consequently they were not included in the in-use preservative efficacy studies.

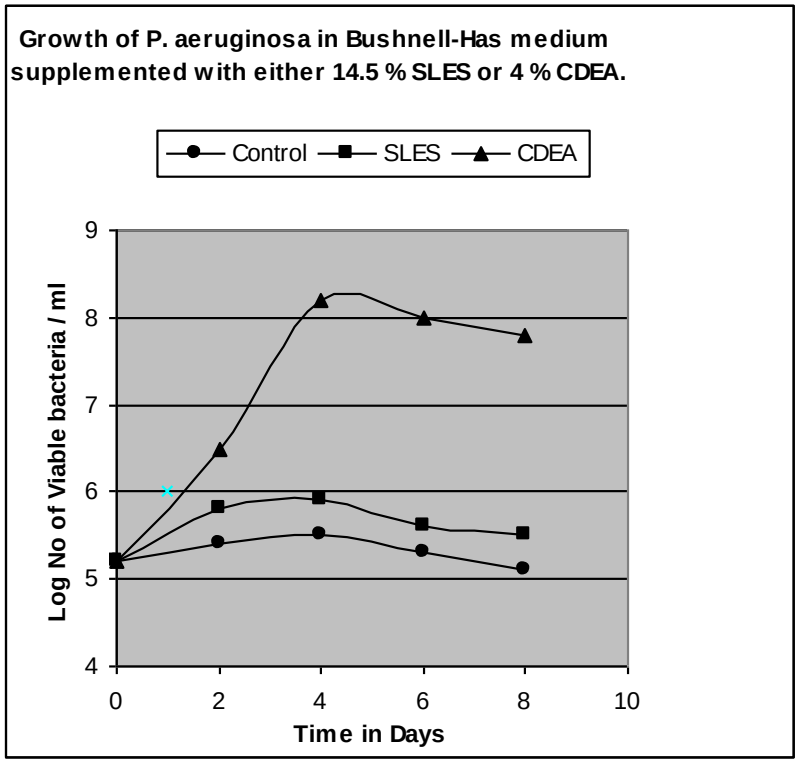
Abdelaziz *et. al* (1989) studied 8 commercial brands of shampoo in Egypt and found that none of the formulations they studied harbored microorganisms in access of 10^4 CFU / ml. Only 15 % of the products revealed bacterial count between 102 to 103 cells / ml. These observations are very close to ours as 15% of their investigated formulations can be considered as out of specifications according to the Jordanian standards.

Table 1. Types and Numbers of Bacteria Isolated from Shampoo Products after Normal Use.

Product code	CFU/ ml	Type of bacteria isolated
B	3 X 10 ^{2}	*Bacillus* sp. and *Serratia* sp.
D	70	*Bacillus* sp, *Staphylococcus* sp*
E	4X10^5	*Bacillus* sp, *Pseudomonas* sp, and *Enterobacter* sp
H	81	*Bacillus* sp and *Staphylococcus* sp*
J	5 X 10^2	*Bacillus* sp, *Enterobacter* sp. and *Pseudomonas* sp
K	2 X 10^4	*Bacillus* sp, *Serratia* sp. and *Pseudomonas* sp.
M	4 X 1o^3	*Bacillus* sp, *Staphylococcus* sp*and *Enterobacter*

* Coagulase negative Staphylococci

Figure 1. Growth Pattern of *Pseudomonas aeruginosa* ATCC 9027 in Mineral Salts Medium (Busnell-Hass) Supplemented with either 14.5 % SLES or 4 % CDEA



In comparison, 18.75% of the shampoo products investigated in this work was found to be out of microbiological limits. These results are not surprising as microbial contamination of current cosmetics particularly shampoos were encountered in various countries (Campana *et al.*, 2006; Lundov *et al.*, 2008).

Unless they are properly preserved, cosmetics provide microorganisms with adequate environments for their

growth. Preservatives are incorporated into formulations to maintain the microbial load in these products to a safe and acceptable level. In order to establish the preservative efficacy of a given formulation, a challenge test is performed (ISO/WD 11930: 2008). This test provides information's regarding the capacity of the preservative system to cope with the actual challenge to which the formulation is usually exposed during use (Russell 2003; Campana, *et al.*, 2006). Another inference from the challenge test is to allow volunteers to use the product for a certain period of time and then test the product for the presence of microorganism (Brannan and Dille, 1990). This is exactly the approach which was adopted in this investigation.

Table 1 shows that after normal use 7 (53.9%) out of the 13 brands harbored viable microorganisms, 2 (15.4%) were within the acceptable limit ($<10^2$ CFU / ml) and 5 (38.5%) were out of limit. These findings cannot be compared with any other work as there is no published literature directly related to post use microbiological quality of commercial shampoo brands. The closest to this work was that performed by Brannan and Dille (1990) who investigated a prototype shampoo formulation, containing no preservatives. These authors established that dispensing closure used for shampoo containers played an important role in protecting cosmetics from in-use microbial contamination. However, detection of *Pseudomonas* sp. and *Serratia* sp. in the returned post use shampoos is consistent with those reported by Brannan and Dille (1990), the difference being in the recovery of *Bacillus* sp. and coagulase negative Staphylococci; these organisms were present in our study but absent in theirs. Nevertheless, the later two bacterial types were isolated from commercial brands marketed in Egypt (Abdelaziz *et al.*, 1989).

The isolation of a variety of gram negative and positive bacteria from 53.85% of post use shampoos raise the obvious question of why preservatives in these products failed to deal with consumers challenges although they were definitely effective when containers were just opened. It is feared that the majority of these products was preserved with formaldehyde (formalin) which has the tendency to evaporate when the container is opened and thus leaving the product without preservation. However, formaldehyde in low concentration is still used for the preservation of cosmetics; high amounts of this compound could be extremely toxic (Rivero and Topiwala, 2004; Yazar *et al*., 1010; Lundov *et al*., 2010). The frequency of use and concentration of formaldehyde in cosmetics manufactured in Jordan is worth a comprehensive investigation.

It is assumed that microbial growth in cosmetics is contingent on the ability of the contaminant to utilize product formulation as carbon and energy source. The range of chemicals used in shampoo formulations is so versatile and thus contaminants will always find chemically needed growth requirements in this man made habitat. SLES is added to shampoos as a primary surfactant while CDEA is employed as a foam booster and both are used in large amounts. It has long been argued that anionic surfactants such as SLES may exhibit antimicrobial properties but unfortunately this argument has not been supported by experimental data (Bryce and Smart, 1965).

It is evident from Figure 1 that SLES at a concentration of 14.5% in mineral salts medium was not inhibitory to *P. aeruginosa* ATCC 9027. In the contrary, almost 7 fold increases in the initial number of the test organism was observed after 2 days of incubation as compared to the viable cells detected in the control medium which was devoid of any carbon source. It is important to note that the concentration of active matter in the commercial grade of SLES used was 70%. Therefore the slight growth obtained could be attributed to the available impurities within the compound and not to the surface active agent itself. On the other hand, *P. aeruginosa* ATCC 9027 was very prolific in mineral salts medium supplemented with 4% CDEA as amain source of carbon and energy. Total amide content in the commercial CDEA was a minimum of 96%.

In this context, it is worthwhile to refer to the statement given by Scott and Gorman (1992) which says that anionic and non-ionic surface active agents have strong detergent properties but exhibit little or no antimicrobial activity. This paper has clearly shown that while CDEA was readily utilizable by Pseudomonad, SLES showed no inhibitory effect against the same organisms.

In conclusion, shampoo preparations based on SLES and CDEA provide microorganisms with environment conducive for their growth and will remain susceptible to microbial attack during use. Manufacturers of these products should use adequate preservative systems, capable of dealing with contaminants that are likely to gain entrance into the product during the production process or normal use by consumers. Cosmetic companies in Jordan should pay special attention to this problem before gaining the confidence of the public.

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