BIOCHEMICAL AND MOLECULAR GENETICS IDENTIFICATION OF SOME *Serratia* ISOLATES

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Serratia sp. has short straight rods and faculatitively anaerobic gram-negative bacteria, which are found in soil, water and air. Their colonies are most often opaque. Somewhat iridescent and white, pink or red in color (Richared *et al.,* 1995). They usually produced extracellular enzymes. This could hydrolyze DNA, lipids, and proteins but not starch. Only rare strains fail to produce one or more of these extracellular enzymes. All species, except *S. odorifera*, can hydrolyze Tween 80; also Chitin is hydrolyzed by all species except *S. rubidaea* and *S. odorifera* (Grimont *et al.,* 1978).

Since 1972, DNA homologies and many intense biochemical comparison studies with other groups and cultures have possible to made in order to discrimination between species of the genus *Serratia*, including *S. odorifera, S. liquifaciens, S. rubidaea, S. ficaria, S. pymuthica, S. fonticola and S. marcescens* (Ewing, 1986).

Over the last 30 years, the genus *Serratia* has become important of its because many species used in biocontrol and pigment production. There have been many reports concerning the identification, antibiotic susceptibility, pathogenicity, epidemiological investigations and fingerprinting of this organism.

Accurate identification is important in defining outbreaks. The API 20E system is one of these methods widely used; it is not individually satisfactory (Hejazi and Falkiner, 1997). Antibiotics used to treat *Serratia* infection include beta-lactam agents, aminoglycosides and fluoroquinolones and a variety of different resistance mechanisms, typing methods used to study the epidemiology of *Serratia* include biotyping, bacteriocin typing, phage typing, plasmid analysis, polymerase chain reaction amplification of enterobacterial repetitive intergenic consensus sequences (ERIC-PCR).

Serological typing has also been used and this method seems to be a suitable first-line typing method for *S. marcescens*, although some strains remain untypable. RAPD-PCR has also been applied to a small number of isolates and seems to be a promising method, for rapid monitoring (Liu *et al.,* 1994).

The purpose of this study is identification of some predominant gram-negative pigmented bacteria isolated from soil. Using API 20E, the protein finger-printing, antibiotic resistant and molecular genetics techniques are used for dis-crimination among isolated *Serratia*.

MATERIALS AND METHODS

Samples collection

Three isolated from bionematocide produced by ACGEB (Nematose 105) (ACGEBSer1, ACGEBSer4 and ACGEBSer5) and remend bacteria isolated from soil of tomato plants growing in Elkhatatba, Egypt using chitin-amended Luria broth agar medium (LBCA). These isolates were purified and stored as freeze-dried samples. The pure single colony was routinely cultured at 28°C on the same medium.

Identification of Serratia sp

The major identification tool used in this study was the API 20E system (Hejazi and falkiner, 1997). The obtained gram-negative pigmented pure culture was subjected to identify using API sys-tem. For complete identification further tests are required.

Bacterial isolates, media and culture conditions

Twenty three *Serratia* isolates (ACGEBSer1 to ACGEBSer23) are used in this study. Medium LBCA was used for *Serratia* growth. It contains 10 g of tryptone, 5 g of NaCl, 5 g of yeast extract, 1.8 g of colloidal chitin, and 15 g of Bacto-agar (Difco) in 1 liter of distilled water. All isolates were stored as frozen stocks at -80°C in a PG medium (1% peptone and 2% glycerol) containing 50% glycerol. The working cultures were prepared from those stock cultures by two consecutive transfers into PG medium. Single-colony was obtained each time for purification.

Antibiotic sensitivity

The isolates were subjected to antibiotic resistance screening by disc diffusion method. For this purpose, lawn of each isolate was made on PG agar plates with the help of ethyl-wire- loop. Then commercially available antibiotic discs (fourteen antibiotics having different mode of actions such as cell wall synthe-sis inhibitors, membrane permeability alternatives, protein synthesis inhibitors and DNA synthesis inhibitors) were placed on previously prepared lawn of culture to be tested. The inoculated plates were incubated at 37°C for 24 h, then presence or absence of inhibition zone around the antibiotic discs was observed (Cheesbrough, 2000).

Extraction of total proteins and SDS-PAGE

Bacterial cultures (1.5 ml of 72-hr-old culture) were harvested by centrifuga-tion (10000xg, 5 min). The obtained pellet was washed with distilled water and recentrifuged. A volume of 100 µl of 2X Buffer, 10 µl lysozyme and 5 µl of 10

mM phenyl-methane-sulfonyl-fluoride as a protease inhibitor (Sigma) was added, then incubated in water bath (37°C) for 30 min. The samples were heated for 5 min in boiling water bath, and suddenly frozen at -70°C (20 min). Each sample was centrifuged for 5 min at 10000xg. Eighty ul from each sample was transferred into a new 1.5-ml Eppendorf tube. Then, 20 µl sample treatment buffer (125 mM Tris-HCl, pH 6.8; 2 % (w/v) SDS, 8 % (v/v) glycerol, 0.0001 % (w/v) bromophenol blue and 5 % (v/v) ß-mercaptoethanol was added. The whole mixture was vortexed to ensure good homogenization. To obtain cell-free extracts, such prepared samples were kept on a boiling water bath for 4 min, and again recentrifuged (10000xg, 10 s) in order to remove any accidentally-remaining whole cells or their fragments (Robert *et al.*, 2001). SDS-PAGE was performed by the method described by Laemmli (1970) and modified by Hames (1995).

Repetitive extragenic palindromic (REP) and enterobacterial repetitive inergenic consensus (ERIC) PCR

Four primers were used in this work with the following nucleotide sequence: REPIR-I 3`-CGG ICT ACI GCI GCI III-5`, REP2-I 5`-ICG ICT TAT CIG GCC TAC-3`, ERICIR 3`-CAC TTA GGG GTC CTC GAA TGT A-5` and ERIC2 5`-AAG TAA GTG ACT GGG GTG AGC G-3; primers were synthesized by Genemed Synthesis Inc (USA). The primers have been described by Versalovic *et al*. (1991). The PCR cycles were as follows: 1 cycle at 95°C for 6 min; 30 cycles at 94°C for 1 min, 56°C for 1 min, and 65°C for 5 min; 1 cycle at 65°C for 12 min; and a final step at 4°C. All the PCRs were carried out in a 25µl volume containing 50 pmol of each primer, 1.25 mM deoxynucleoside triphosphate, 50 ng of template DNA, and 1 U of *Taq* DNA polymerase (Gibco, BRL). Buffers supplied with the enzyme were used according to the manufacturer's directions. The DNA amplification was performed in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400). After the reaction, 8 ul of amplified DNA was separated on 1.5% agarose gels (Sigma), stained with ethidium bromide, and recorded with Gel Doc system (BioRad.). All PCRs were independently performed at least three times.

RESULTS AND DISCUSSION

Bacterial identification

The present work is planed to identify 23 *serratia* isolates. They were isolated from soil and identified on the bases of morpho-cultural and biochemical considerations. API 20 E kit was used for the final identification of the isolates. The isolates were allocated to *Serratia* with high identification probability 99.9%. The isolates divided into two species. Nine of them (ACGEBSer1, ACGEBSer4, ACGEBSer5, ACGEBSer6, ACGEBSer8, ACGEBSer10, ACGEBSer12, ACGEBSer13 and ACGEBSer22) were *Serratia marcescens* and the remainder fourteen isolates (ACGEBSer2,

ACGEBSer3, ACGEBSer7, ACGEBSer9, ACGEBSer11, ACGEBSer14, ACGEBSer15, ACGEBSer16, ACGEBSer17, ACGEBSer18, ACGEBSer19, ACGEBSer20, ACGEBSer21 and ACGEBSer23) were *Serratia liquefaciens*.

Antibiotic resistant patterns

Antibiotic resistant is one of the most important criteria for persistence and competition of target bacteria with other bacteria in habitat, *i.e.* soil for bacteria that used in compete soil fungal diseases (Schroth *et al*., 1991). Furthermore, antibiotic pattern is important character for identification and scrimination of bacteria. Also antibiotic resistance could be used as a selectable marker. For these reasons, the isolated *Serratia* are tested for resistance of several types of antibiotics that could be produced from soil microorganisms that compete of them.

Discs of penicillin G, amoxicillin, claforan and duricef (peptidoglycan synthesis inhibitors), streptomycin, tetracyclineand, erythromycin (block translation), nebcin, neomycin and vibramycin (faulty protein synthesis), garamycin (inhibits protein synthesis), bacitracin (inhibits dephosphorylation of lipid), flagyl (generate toxic O_2 radicals) and rifadin (inhibits RNA) were used for this test. Table 1 showed the resistance pattern to commonly used antibiotics. All isolates were found to be resistant to one or more of the antibiotics. The streptomycin is selectable markers for isolate ACGEBSer4 and ACGEBSer14. All isolates are resistant for all antimicrobial agents that inhibit peptidoglycan synthesis (penicillin G, amoxicillin and duricef), inhibits dephosphorylation of lipid (bacitracin) and generate toxic oxygen (flagyl).

Resistance to aminoglycosides in gram negative bacilli is often mediated by ß-lactamases which are unaffected by exposure of the bacterium to the potential drugs interestingly, (Shafran, 1990) found the resistance to penicillin G, amoxicillin is more frequent in his study. It has been argued that there is a direct relation between the antibiotic used and the frequency and kinds of antibiotic-resistant strains (Kupersztoch, 1981). The resistance to antimicrobial agents can readily be transferred among bacteria by transmissible elements plasmids (Neu, 1994). These resistant organisms can pass their resistance genes to their offspring by replication or to related bacteria through conjugation (Tomasz, 1994)

Data presented in Table (4) clearly show that neomycin was the most efficiency against the tested *Serratia* isolates followed by streptomycin being 100% and 91.3% respectively. On the other hand nebcin, vibramycin and Claforan gave the same figure against *Serratia* isolates being 86.91% (20 out of 23). On the contrary five among fourteen antibiotics namely flagyl, bacitracin,, pencillin, amoxil and duricef were not effective against all tested *Serratia* isolates.

Biochemical fingerprint

Electrophoretic separation based on SDS-PAGE of cellular proteins (total proteins) was used for identification and/or differentiation of different micro-organisms (Ibrahim *et al.*, 1990; Peter and Bretz, 1992; Hertel, *et al*., 1993; Abdel-Razik, 1998) also used for typing certain *Serratia* bacteria (Lambert, *et al.*, 1987; Arnowski *et al.,* 2001). In the present investigation, SDS-PAGE was used for identification and differentiation of different *Serratia sp.* The gel was scanned and analyzed using Quantity one (Bio Rad). The SDS-PAGE photograph is given in Figs. (2 & 3). In this prospect the SDS-PAGE was successfully used for both the identification and differentiation of *Serratia* sp. The analysis of whole-cell protein extracts of 23 isolates of *Serratia* by one-dimensional SDS-PAGE revealed a set of clearly different profiles. There were fifteen common bands (numbers 4, 7, 8, 11, 12, 13, 15, 20, 21, 22, 23, 24, 27, 31 and 32) with molecular weights approximately 92.79, 80.00, 76.65, 57.72, 55.00, 53.18, 45.00, 33.00, 31.41, 28.50, 27.00, 16.86, 20.00, 16.24 and 15.00 kDa, respectively.

The negative bands exist in isolate number Ser.15, which was approximately 84.72 kDa whereas isolate number 13 have two negative band numbers 25 was approximately 24 kDa and band number 30 was approximately 17.17. On the other hand positive band number 26 existed in three strains were Ser 17, Ser 14 and Ser 9. The band number 5 has approximately 88 kDa was absent in two strains Ser.2 and Ser.4 and band number 18 has approximately 36.00 kDa was absent in two strains Ser.21 and Ser.22.

The computer-assisted numerical processing of these patterns using cluster analysis with Euclidean distances yielded a dendrogram, which consisted of several groups at similarity levels ranging from 15 to 84.2 % of the maximum distance. Based on the electrophoretic mobility of cellular proteins, the *Serratia* strains studied were grouped into three main clusters (I, II and III). Each group was additionally discriminated into minor clusters grouping those protein profiles of the highest similarity. Within group I, represented by 2 isolates were distinguished. Those strains that have already been classified as same species, like *Serratia marcescens* within Group II, which showed the highest similarity to group I. The results presented here led to the *Serratia liquefaciens* into several clusters, which is a good reflection of the heterogeneity of this species as described in the literature (Arnowski *et al*., 2001). (Figs. 1 & 2).

DNA Fingerprinting based on repetitive extragenic palindromic (REP) and Enterobacterial repetitive inergenic consensus (ERIC)

PCR amplification of extracted DNA with REP and Eric primers revealed species-specific patterns of amplification products for the various *Serratia* isolates (Figs. 3 & 4). As described previously in other bacteria for which this technique has

been used, related organisms showed some similar bands. ERIC-PCR amplification with degenerate primers generated fingerprint paterns having approximately 7 or 8 bands per isolate (Fig. 3). These bands ranged in size from ≈44bp to ≈727bp. Strain *Serratia liquefaciens* (ACGEBSer15) have one positive band (about ≈660bp).

REP-PCR amplification with degenerate primers generated fingerprint paterns having approximately 7 bans per isolate (Fig. 4). These bands ranged in size from ≈53bp to ≈885bp two common bands were present in all strains (about ≈ 53bp, ≈607bp). Strain *Serratia marcescens* (ACGEBSer8) has one positive band (about ≈115 bp). While strains (ACGEBSer3), (ACGEBSer10), (ACGEBSer12) and (ACGEBSer13) have a positive band (≈652bp). Strains (ACGEBSer3), (ACGEBSer10), (ACGEBSer11), (ACGEBSer12) and (ACGEBSer13) have one positive band (≈512bp). The similarity of rep-PCR and Eric-PCR fingerprints for these isolates suggests that they may come from the same origin or there are little genetic diversity has occurred over the years Restricted diversity has been described for other bacteria (*e.g Salmonella spp*., and *Legionella peneumophila*) (Georghiou *et al*., 1995).

SUMMERY

In the present study, 23 of *Serratia* isolates were isolated and identified using API 20E test, Antibiotic resistant patterns, SDS-PAGE, DNA fingerprinting based on Repetitive extragenic palindromic (REP) and, Enterobacterial repetitive inergenic consensus (ERIC) was carried out. The isolates were allocated to *Serratia* with high identification probability 99.9%. Out of this isolates, there are 9 isolates identified as *Serratia marcescens* and 14 identified as *Serratia liquefaciens.*

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Table (1): Antibiotic resistant pattern of different antibiotics among *Serratia* isolates.

Antibiotic	Properties of antibiotic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Rifadin	Inhibits RNA	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-
Flagyl	Generate toxic o_2 radicals	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bacitracin	Inhibits dephosphorylation of lipid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Garamycin	Inhibits protein synthesis	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	-	-
Neomycin	Faulty protein synthesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nebcin	Faulty protein synthesis	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Vibramycin	Faulty protein synthesis	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
Claforan	Inhibit peptidoglycan synthesis	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Pencillin	Inhibit peptidoglycan synthesis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amoxicillin	Inhibit peptidoglycan synthesis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Duricef	Inhibit peptidoglycan synthesis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythromycin	Block translation	-	+	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	-	-
Streptomycin	Block translation	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Tetracycline	Block translation	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	+	+

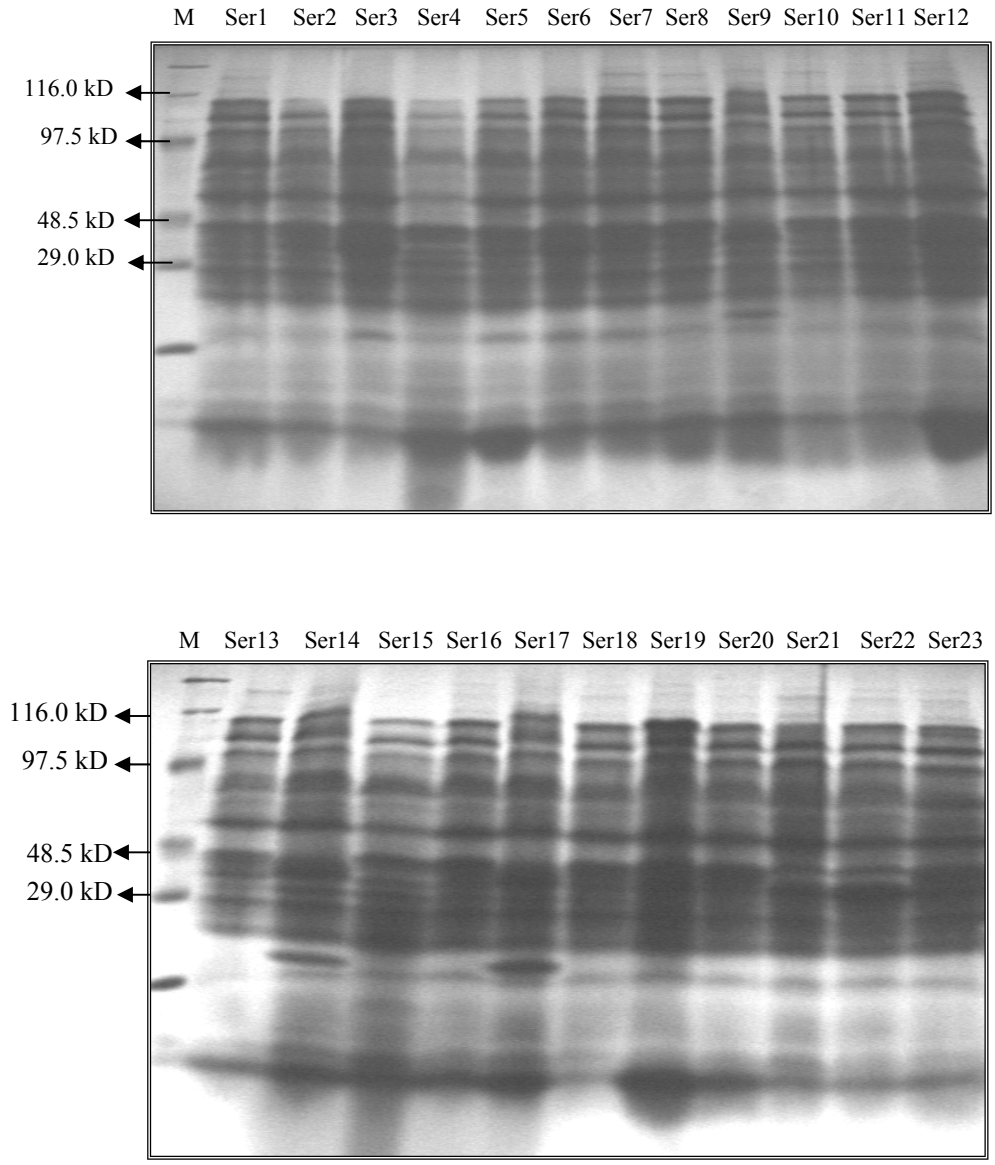

Fig. (1): SDS-PAGE of total cell-free proteins extracted from the tested *Serratia* isolates.

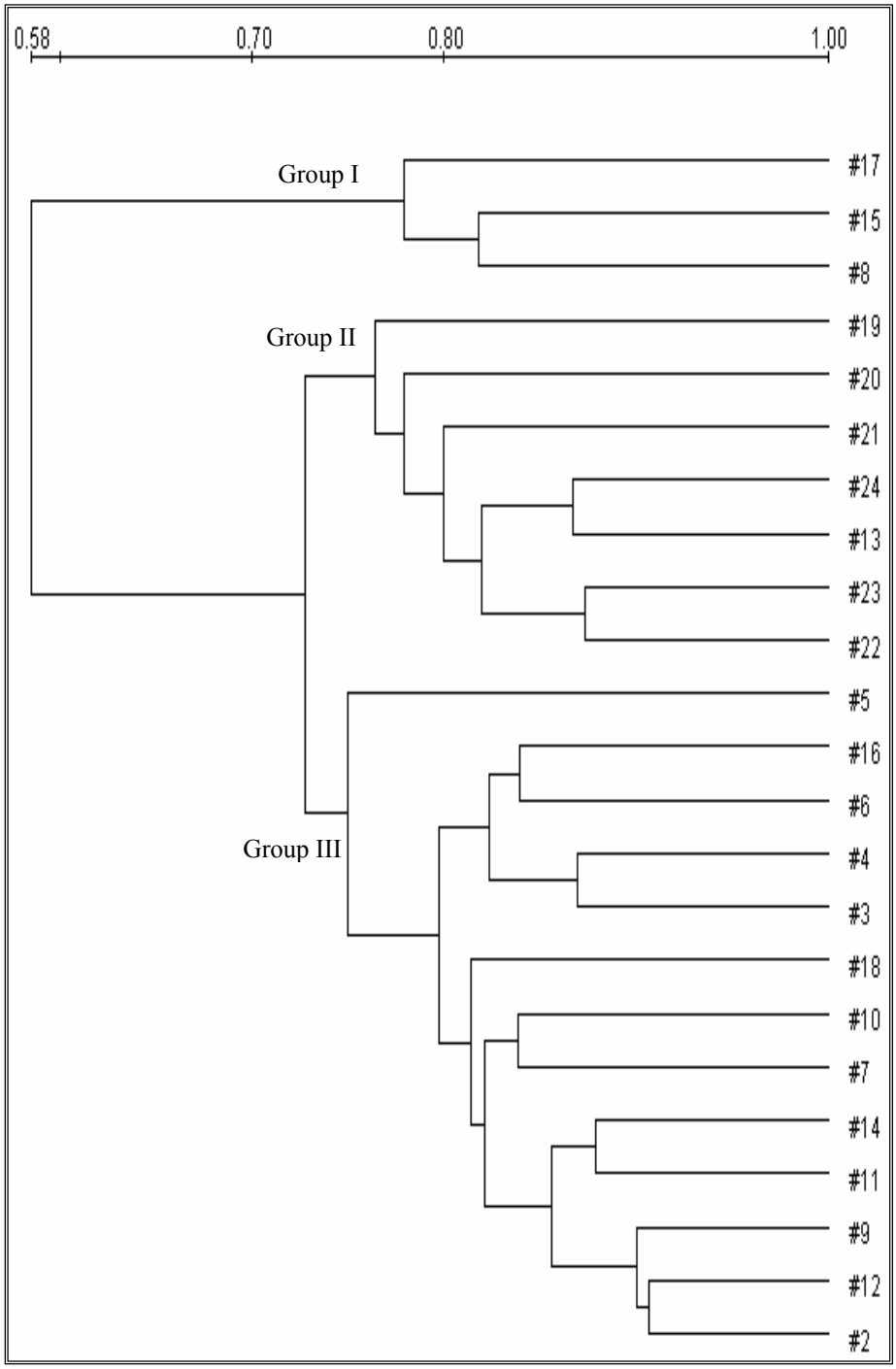

Fig. (2): Grouping of the *Serratia* isolates studied using cluster analysis (Euclideandistances, Ward's amalgamation algorithm) based on whole-cell protein.

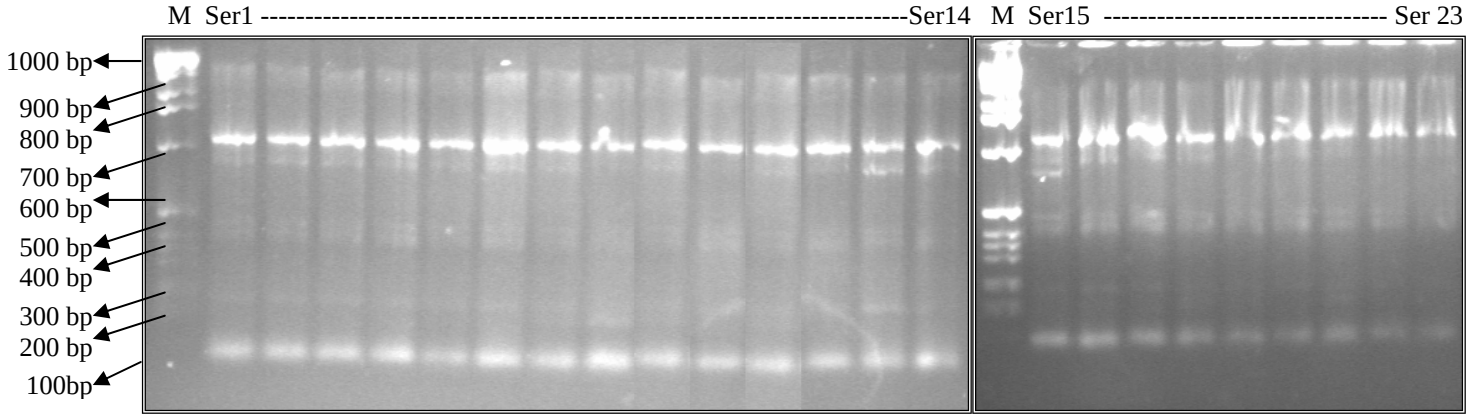

Fig. (3): DNA polymorphism based on ERIC primers for 23 *Serratia* strains and M, refers ferments marker.

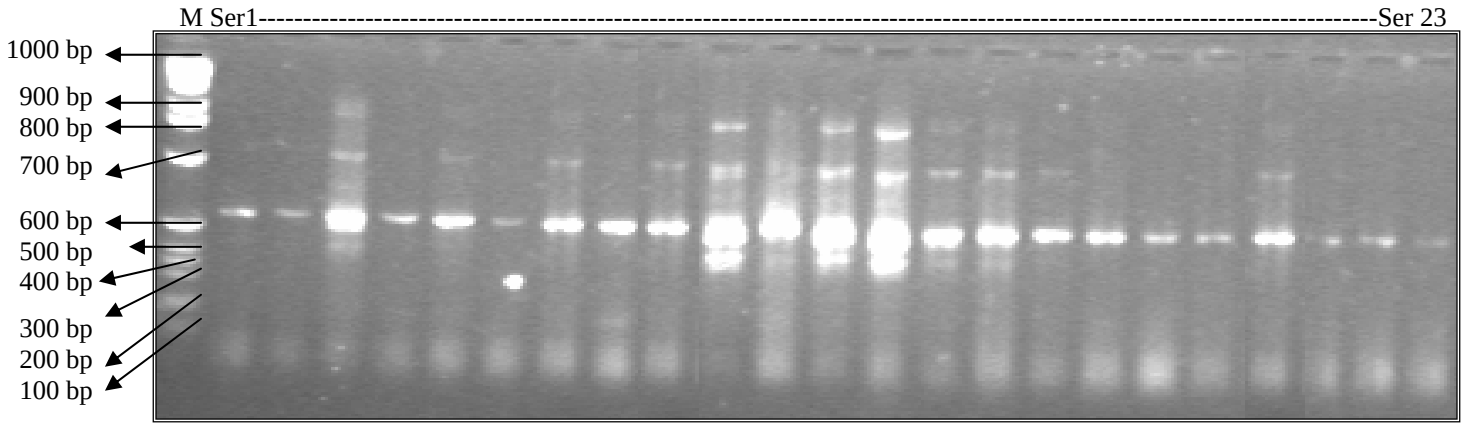

Fig. (4): DNA polymorphism based on REP primers for 23 *Serratia* strains and M, refers ferments marker.