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MICROBIAL QUALITY AND MOLECULAR IDENTIFICATION OF PATHOGENIC BACTERIAL STRAINS COLLECTED FROM RAW CAMEL'S MILK IN TAIF REGION

Mahmoud F. Samy[1,3], El-Halmouch Yasser[2], AL Zhrani M. Othman[1] and Sayed A Amer[4,5]

[1]Biotechnology Department, [4]Biology Department, Faculty of Science, Taif University, Taif 21974, Saudi Arabia

[2]Botany Department, Faculty of Science, Damanhour University, Damanhour 22511, Egypt

[3]Departement of Dairy Technology, Food Technology Research Institute, Agricultural Research Centre, Giza, Egypt

[5]Departement of Zoology, Faculty of Science, Cairo University, Giza, Egypt

ABSTRACT

Sixty-four samples of raw camel's milk were collected from Jia, Oshera, Bani-Sa'ad and Al-Roduv at Taif area in different seasons. They were analysed for microbial quality. Molecular and biochemical identification for all isolated pathogenic bacteria were done. Total bacterial, yeasts and moulds counts increased significantly in summer, while they decreased in winter. The total viable counts of bacteria, yeasts and molds in Jia were 2.9×10^5 and 1.7×10^2 during Summer and 1.34×10^5 and 0.31×10^2 in winter, respectively. In Al-Roduv they were 0.9×10^5 and 0.94×10^2 in Summer, 0.65×10^5 and 0.64×10^2 in winter, respectively. The ribosmal 16S rDNA gene was completely sequenced for the isolated strains and their sequences were used with their counterparts of other related taxa to molecularly identify the isolated strains. The selected pathogenic bacteria were identified as *Proteus mirabilis, Escherishia coli, Serratia nematophila* and *Bordetella petrii* based on their morphological, biochemical and molecular characterisation. They represent 25, 35.93, 9.37 and 18.75 per cent of the total microbial count, respectively.

Key words: Bacterial count, camel, microbial quality, milk

The hyieginic conditions of the milking place, the excretion from the udder of an infected animal and quality of water used on the farm, may influence the microbiological quality of milk products (Amaral *et al,* 2003, Angulo *et al,* 2009). Physico-chemical, microbiological, hygienic and sanitation measures have been deployed by the industry to test and verify the quality of milk (Guerreiro *et al,* 2005). Raw Milk (RM) often contains microorganisms, which may cause food borne diseases (Adesiyun *et al,* 1995; Steele *et al,* 1997; Headrick *et al,* 1998). The number and types of microorganisms in milk immediately after milking are affected by animal and equipment cleanliness, season, feed and animal health (Rogelj, 2003). Bacterial contamination of raw milk can be happened from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al,* 2008). It is suggested that the differences in feeding and housing strategies of cows may influence the microbial quality of milk (Coorevits *et al,* 2008). Microbes like *listeria monocytogenes*, Salmonella, Campylobacter, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* and thermotolerant coliforms, especially *Escherichia coli* are the most common contaminant of raw camel's milk (Chye *et al,* 2004; Mhone *et al,* 2011).

The total viable count of bacteria is one of the main criteria used to evaluate the classification and processing of dairy products (Chye *et al,* 2004; Mhone *et al,* 2011). Although freshly drawn milk from animals may possess temporary 'germicidal' or 'bacteriostatic' properties, growth of microorganisms is inevitable unless it is processed by freezing, heat treatment or irradiation (Murphy and Boor, 2000; Saeed *et al,* 2009). Daily production, eventual marketing and sale of milk require special consideration to ensure its delivery to the market in hygienic and acceptable condition. In developing countries, outlets for the purchase of milk are numerous but most operate under unsanitary conditions and are not adequately monitored or regulated (Food and Agriculture Organisation, 1990, 2003). Under such conditions the food-borne zoonotic risk posed by milk and dairy products is of great public concern (Vanden Berg, 1988).

Several molecular studies have been conducted to identify the pathogenic bacteria found in the

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raw camel milk. As repesentatives of these studies Benmechernene *et al* (2014) identified the genomic characterisation of *Leuconostoc mesenteroides* strains isolated from raw camel milk in 2 Southwest Algerian Arid Zones. Akhmetsadykova *et al* (2015) studied the molecular biodiversity of lactic acid bacteria in raw and fermented camel's milk. Moumene *et al* (2016) studied the complete genome sequence of *Lactococcus garvieae* M14 isolated from algerian fermented milk. The present work is conducted to evaluate the microbial quality of raw camel milk from the Taif-region and isolate and identify the pathogenic strains, which are present in camel's milk using molecular and biological methods.

Materials and Methods

Milk Samples

Raw camel milk samples were collected from different places (Jia, Oshera, Bani Sa'ad and Al-Roduv) of Taif region during lactation period under aseptic conditions in a sterile screw cap tubes, processed within three hours and used for further studies.

Microbiological analysis

Milk samples (25 ml) were diluted in buffered peptone saline (225 ml, 0.5% w/v; peptone; 0.85% w/v; NaCl), mixed in stomacher bag and stomached in Seward stomacher (Seward 400, England) for 2 minutes. In order to quantify the various microbial groups, appropriate dilutions (101-105) were surface plated. Aerobic total plate count (ATPC) was carried out on plate count agar (PCA), incubated at 32°C for 72h (Marshall, 1992). For aerobic mesophilic spore count (AMSC), the milk was heat-shocked at 80°C for 10 min to destroy vegetative cells. After being cooled in an ice bath, the milk was immediately plated on plate count agar and incubated at 32°C for 48 h (Marshall, 1992). Total and faecal coliforms were determined by MPN method according to US standard method (Federal Register, 1990). Moulds and yeasts were enumerated on potato Dextrose Agar (PDA) acidified by lactic acid (Oxoid, SR21). For detection and isolate *salmonella* spp, a portion of 25 ml of milk was pre-enriched in 225 ml of buffered peptone water at 37°C for 24h. Then, 1 ml of pre-enrichment sample was incubated in 10 ml Cystine Selenite broth and Rappaport-Vassiliadis broth at 37°C for 24h. Selective enrichments were then streaked onto Bismuth Sulphite, xylose lysine Desoxycholate (XLD) and Hekton entreic agars. All selective media were incubated at 37°C for 24h. Typical colonies were examined by microscope, characteristics of growth on lysine iron agar, urease production and then tested with *Salmonella polyvalent* (Salmonella latex test, Oxoid FT0203).

Biochemical characterisation

Inculated plates were incubated at different temperature ranging from 5 to 55°C. The pH growth range was determined by streaking each inoculum onto the surface of preferred medium adjusted at different pH values ranged from 5 to 11 adjusted by HCl or NaOH. The ability to grow anaerobically was evaluated on solid medium incubated in jars with the GasPak envelops (BBL). Haemolysis was studied in solid Mueller Hinton (MH) medium supplemented with 5 % (v/v) defibrinated sheep blood. Oxidase reaction was performed according to Kovács (1956). Catalase was determined by adding 10 volumes of 3% H_2O_2 to each strain culture on their solid medium. Indole production was tested in liquid MH medium using Kovács' reagent (Kovács, 1928). Methyl red and Voges-Proskauer were tested using methyl red and Barritt's reagent (Barritt, 1936), respectively. Starch agar was used to test the ability of an organism to produce certain exoenzymes, including a-amylase and oligo-1,6-glucosidase (Srivastava and Baruah, 1986; Mishra and Behera, 2008). Cultures were inoculated into tubes of nutrient gelatin (nutrient broth, 100ml; Difco gelatin, 12 g; pH 7.0) and incubated at 37°C for 14 days. After they had been cooled to 4°C, they were observed for liquefaction of the gelatin. Casein hydrolysis was indicated by a clear zone around bacterial growth on solid MH medium plus an equal quantity of skimmed milk (Carpana *et al,* 1995). Cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo-Red agar media (Lu *et al,* 2004). Chitin hydrolysis was measured by the halo diameter of enzyme diffusion on the chitinase production medium (Gao *et al,* 2015). Production of acid from carbohydrates was determined by the methods of Gordon *et al* (1974). Finally, Urea hydrolysis was detected on Christensen's medium (Christensen, 1946).

Antimicrobial susceptibility tests

Antimicrobial susceptibility of the selected bacterial strains was assayed using the diffusion agar method (Bauer *et al,* 1966). The antimicrobial compounds (MASTRING-S^{TM}) used in this study included; Ampicillin (25µg), Tetracycline (30µg), Chloramphenicol (30µg), Levofloxacin (5µg), Flucloxacillin (5mcg), Tobramycin (10mcg), Ofloxacin (5mcg), Norfloxacin (10mcg), Cefotaxime (30µg), Imipenem (10µg).

DNA Isolation from pathogenic bacteria isolate

1.5 ml of broth was taken in a microfuge tube and centrifuged at 5000 rpm for 15 min. The supernatant was discarded. To the pellet, 1.5 ml of broth was added and again centrifuged at 8,000 rpm for 10 min. 467 µl of Tris-EDTA (TE buffer), 50 µl of 0.5% lysozyme and 30 µl of 10% Sodium Dodecyl Sulfate (SDS) was added. Then 3 µl of proteinase k was added (20 mg/ml). It was mixed well and incubated for 1 h at 37°C. After the incubation, equal volume of phenol: chloroform (25:24) mixture was added and mixed well by inverting the tubes gently until the phases are completely mixed. Then it was centrifuged at 8,000 rpm for 10 min. After centrifugation, the upper aqueous phase was transferred to a new tube and 1/10 volume of sodium acetate was added. Then 0.6 volume of isopropanol was added and mixed gently until the DNA gets precipitated and then centrifuged at 8,000 rpm for 10 min. To the pellet, 70% ethanol was added to the pellet and centrifuged at 5,000 rpm for 10 min. 20 µl of TE buffer was added and stored in ice cold condition (Sambrook *et al,* 1989) and analysed by Agarose gel electrophoresis.

PCR and sequencing 16S rDNA

Both the forward primer 5`-27F 5′ (AGA GTT TGA TCM TGG CTC AG) 3′ and the reverse primer 5`-1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3′ were used for amplifying the complete 16S rDNA gene for the isolated DNAs of the bacterial strains. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µl reaction mixture by using a EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles of 95°C for 1 minute, 55°C and 72°C for 1 minutes each were performed, finishing with a 10- minutes step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing Kit. The sequencing primers were 785F 5′(GGA TTA GAT ACC CTG GTA) 3′ and 907R 5′(CCG TCA ATT CMT TTR AGT TT) 3′. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analysed by ABI Prism 3730XL DNA analyser (Applied Biosystems, Foster City, CA).

The ribosomal 16S rDNA gene has been sequenced for the current bacterial strains. These data have been treated with Blastn program (http://www.ncbi.nlm.nih.gov) for searching for their closely related strain sequences already found in the Genbank database. Sequences with identity of 99 to 95 % to these two strains have been collected from the database. The collected data were used for phylogenetic analyses after unalienable and gap-containing sites were deleted (1422 bp in total). The aligned nucleotide sequences can be obtained from the corresponding author upon request. The analyses were done by maximum-parsimony (MP) and neighbor-joining (NJ) by heuristic searches with the TBR branch swapping and 10 random taxon additions. Bootstrap replications were adjusted for both methods to be 10000 replications. The maximum-likelihood method in PAUP* 4.0b10 (Swofford, 2003) was also used with NNI branch swapping and axis taxon additions and 200 bootstrap replications. The general reversible model (GTR+I+G) and parameters optimised by Modeltest 3.0 (Posada and Crandall, 1998) were used.

Result and Discussion

Camel's milk collected from four different ecological areas, that include Oshera, Al-Roduf, Bani Sa'ad and Alkor. Many authors described the ability of camel milk to inhibit the growth of many bacterial spp. due to the lytic action of lysozyme and lactoferrin contained in camel milk (Al- Majali *et al,* 2007; Wernery 2003; El-Agamy *et al,* 1992). Four other different pathogenic bacteria i.e., *Proteus mirabilis*, *E. coli*, *Serratia nematodiphila* and *Bordetella petrii* have also been detected. Camel milk still represents a significant source of infection for human (El-Ziney and Al-Turki, 2007; Matofari *et al,* 2007; Vanegas *et al,* 2009). Regarding to the overall prevalence of tested samples by using bacteriological isolation and biochemical identification, results revealed that an overall prevalence and hygienic quality of camel's milk were determined by total viable count of bacteria and number of yeasts and molds (Table 1).

The total viable count of bacteria varied significantly according to the site and the season of collection. Total viable count of bacteria ranged from 1.34 to 2.9 $\times 10^5$, 1.2 to 1.03$\times 10^5$, 1.9 to 1.36 $\times 10^5$ and 0.9 to 0.65$\times 10^5$ in Jia, Oshera, Bani-Sa'ad and Al-Roduv, respectively (Table 1). The highest count was obtained for Jia at summer season (Table 1). These results agree with Younan (2004) who reported that the number of bacteria ranging between 100 to 10,000 cell/ml in Kenya. On the other hand Al–Mohizea (1986) found that the aerobic plate count exceeded 10^5cfu/ml in 13 samples and averaged 2.2x10^5 cfu/ml in camel's

raw milk in Riyadh City. Moreover in Ethiopia, Semereab and Molla (2001) reported that the bacterial count ranged from $0.4x10^5$ to 10^5 cfu/ml. In addition El-Demerdash and Al-Otaibi (2012) reported that the total bacterial count of raw camel milk samples collected from different zones ranged from 1.3×10^3 to 1.3×10^6 cfu/ml. Total viable counts (TC) in milk more than 10^5 cfu/ml. These gave an evidence of the bad hygienic condition during milk production (Al–Mohizea, 1986). Our resultes indicated that the number of bacteria in summer were higher than those of Spring, Autumn and Winter. These results may be due to equipment cleanliness, season, feed and animal health (Rogelj, 2003), air, milking equipment, feed, soil, faeces and grass (Coorevits *et al,* 2008), the hygienic conditions of the milking place, the excretion from the udder of an infected animal and quality of water used on the farm (Amaral *et al,* 2003; Angulo *et al,* 2009; Torkar and Teger, 2008; Parekh and Subhash, 2008). The highest number of bacteria may be due to higher tempreature during storage and distribution in summer. Younan (2004); Swai *et al* (2002); Zelalem and Faye (2006), reported that the differentiation in bacterial count may be due to milk structure, ways of milk collection and environmental conditions. In addition, yeast and mold numbers were varied significantly according to the season and site of collection, the highest number was observed in summer at Jia, Oshera, Bani-Sa'ad and Al-Roduv, they were 1.7, 0.99, 1.24 , 0.94 $\times10^2$ cfu/ml, respectively. While the lowest values recorded at Jia and Oshera in autumn, they were 0.31 and 0.18×10^2 cfu/ml, respectively. On the other hand, yeast and mold counts were 0.19 and 0.38×10^2 cfu/ml in Bani-Sa'ad and Al-Roduv, respectively in winter. These results are in agreement with those of Karmen and Slavica (2008). Who reported that yeasts present in 95.0% of raw camel's milk samples with the mean concentration of 1.7 log10 cfu/ml. In addition, moulds were found in 63.3% of raw camel's milk samples, their mean concentration was 0.6 log10 cfu/ml. In addition they added that isolated mould strains belonged to genera *Geotrichum* (51.5%), *Aspergillus* (33.8%), *Mucor* (5.9%), *Fusarium* (2.9%) and *Penicillium* (2.9%). Numbers of yeasts and moulds in samples of camel's milk collected from Al-Ahsa area varied from 43 to 8.1×10^3 cfu/ml. Omar and Eltinay (2008), found that the rate of isolation of yeasts from all samples were 14.9%. These species were *Candida ciferri* and *Candida guilliermondii*.

Pathogenic bacteria in raw came milk samples

It has been concerned in this study to use special selective media to isolate 4 pathogenic bacteria; *Proteus mirabilis*, *Escherichia coli*, *Serratia nematodiphila, Bordetella petrii*. Raw milk often contains microorganisms, which may cause food borne diseases (Adesiyun *et al,* 1995; Steele *et al,* 1997). All milk samples tested for the presence of *Proteus mirabilis*, *Escherichia coli*, *Serratia nematodiphila*, *Salmonella* spp. and *Bordetella petrii*. Four pathogenic bacteria were isolated from camel's milk samples as shown in table 2.

The results showed that 25% of camel's milk samples contained *P. mirabilis*. These results are in agreement with FAO (2003). Results revealed different overall prevalences of microbes, i.e. *Salmonella* spp. 2.7%, *E. coli* spp. (Marth and Ryser,

Table 1. Microbiological quantity of camel's milk samples collected from Jia, Oshera, Bani-Sa'ad and Al-Roduv.

	Total bacterial count cfu×10^5/ml				Yeasts and molds cfu×10^2/ml			
Sites	**Summer**	**Winter**	**Spring**	**Autumn**	**Summer**	**Winter**	**Spring**	**Autumn**
Jia	2.9^{Aa}	1.31^{Ca}	2.1^{Ba}	1.34^{Ca}	1.70^{Aa}	0.69^{Ca}	1.18^{Ba}	0.31^{Da}
Oshera	1.2^{Ac}	0.99^{Bb}	1.04^{Bc}	1.03^{Bb}	1.24^{Ab}	0.51^{Ca}	0.8^{Bab}	0.18^{Da}
Bani-Sa'ad	1.9^{Ab}	0.95^{Db}	1.65^{Bb}	1.36^{Ca}	0.99^{Ac}	0.19^{Da}	0.49^{Cb}	0.8^{Ba}
Al-Roduv	0.9^{Ac}	0.70^{Bb}	0.76^{Bc}	0.65^{Cc}	0.94^{Ac}	0.48^{Cb}	0.48^{Cb}	0.64^{Ba}

Different superscript letters in the same row for each parameter (total bacterial count; yeast and mold) are significantly different at P≤ 0.05. Different letter in the column for each parameter are significantly different at P ≤ 0.05.

Table 2. Pathogenic bacteria detected in raw camel's milk sample.

Bacterial strains of positive samples	Number of samples	Infected samples	Per cent of infection
P. mirabilis	64	16	25
E. coli	64	23	35.93
S. nematodiphila	64	6	9.37
B. petrii	64	12	18.75

1990) 6.48% and *Listeria* spp. Chaibou (2005). Abeer *et al* (2012) stated that 1.08% of *Salmonella* spp. were detected with a prevalence rate ranging from 2.38 - 2.85%, where the lowest rate was detected in Sharqia milk samples. Many *E. coli* strains was detected with a prevalence rate ranging from 5.71 - 7.14% where the lowest rate was detected in Sharqia milk samples while the highest rate was detected with Sinai milk samples. *Listeria* spp. was detected in a prevalence rate ranging from 0- 2.85% while the negative results were detected in Sinai and Aswan milk samples. *E. coli* frequently contaminates food and considered as good indicator of faecal pollution (Dilielo, 1982; Soomro *et al,* 2002; Benkerroum *et al,* 2004). Presence of *E. coli* in milk products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard. Enteropathogenic *E. coli* can cause severe diarrhoea and vomiting in infants and young children (Anon, 1975). The negative samples of most pathogenic bacteria may be due to the activity of protective protein such as lysoyeme, lactoferrin, lactoperoxidase, immunoglobulin G and A of camel's milk, as stated by Barbour *et al* (1984).

Description of strain Proteinus mirabilis

P. mirabilis strain showed a gray colour colony, rod-shaped cells, motile and non-sporulated (Table 3). The isolate was facultatively anaerobic for growth. Optimal temperature was 30°C. The temperature range was 30-37°C. pH range was between 7.5 up to 8.5. Methyl red, haemolytic activity, catalase and nitrate reductase were positive. *P. mirabilis* tested negative for the gram reaction, oxidase, Voges-proskauer, d- arabinose, l- arabinose, mannose, maltose and manitol. It was able to utilise, starch, cellulose, urea, arginine, ornithine and gelatin as a sole carbon source. It was unable to utilise casein. The isolate tested against 10 antibiotics listed in table 3. The results obtained showed that the isolate was resistant to flucloxacillin. The antibiotics inhibited the growth of *P. mirabilis* were ampicillin, cefotaxime. levoflxacin, chloramphenicol, tobramycin, tetracycline, ofloxacin, norfloxacin and imipenem, while it was resistant to flucloxacillin.

Description of E. coli

Escherichia coli was rod shaped (coccobacillus) forms shiny, mucoid colonies which had entire margins and were slightly raised. Older colonies had a darker centre, facultatively anaerobic bacteria, non-sporulation, pigmentation was orange red and cells were motile. Optimum temperature was 35-37°C. The temperature range was 21-37°C. pH range was between 6 up to 7. Haemolytic activity, indole production, catalase, nitrate reductase, methyl red were positive. While, it showed negative for the H_2S production, gram reaction, oxidase, Voges-proskauer, acid production from maltose and phenylalanine deaminase. It utilised various sugars including d-xylose, l-xylose, d-glucose, mannose, rhamenose, sucrose, lactose, d-arabinose, l-arabinose and mannitol to produce acid. Both decomposition of starch, cellulose, tryptophan, arginine, lysine and ornithine were positive. It was unable to utilise gelatin, urea and casein as a sole carbon source. Among the tested antibiotics, *E. coli* was resistant to ampicillin and cefotaxime (Table 4).

Description of Serratia nematodiphila

S. nematodiphila has a pink-brown colony, rod shaped cells and pigmentation is red. The cells were motile. The isolate had facultative anaerobic conditions for growth. Optimal temperature was 37°C. The temperature range of growth was 5-40°C. pH range was between 5 up to 9. Optimal pH was 7.2. Gram reaction, indole production, methyl red, sporulation, H_2S production, nitrate reductase and oxidase activities were negative. It showed positive to Voges-Proskauer, haemolytic activity, catalase activity. It had the ability to produce acids from all tested sugars except L-arabinose (Table 3). It was able to utilise starch, gelatin, cellulose, casein, tryptophan, arginine, lysine and ornithine. It did not utilise the urea. Antimicrobial activity tests obtained showed that *S. nematodiphila* varied in its susceptibility to all antimicrobials used. It was resistant to Levoflxacin, Ofloxacin and Imipenem (Table 4).

Description of Bordetella petrii

As shown in table 3 *B. petrii* strain had a creamy white colour colony, pigmention was brown and had anaerobic growth. The biochemical and physiological properties of strain *B. petrii* are summarised in tables 3. It tested positive for Voges-Proskauer, catalase activity and oxidase while negative for motility, gram reaction, methyl red, sporulation, haemolytic activity, indole production and nitrate reductase. However, optimum temperature was observed at 35°C. Optimum pH was 7.9. Strain *B. petrii* unutilised all sugars in table 3 except mannose as sole carbon sources for growth and produced acids from these carbohydrates. *B. petrii* was unable to decompose or hydrolyse gelatin, urea, casein, tryptophan, arginine, lysine, ornithine, starch and cellulose. Strain *B. petrii* was susceptible to all tested antibiotics except flucloxacillin (Table 4).

Table 3. Morphological, biochemical and nutritional characteristics of the selected strains: *P. mirabilis, E.coli, S. nematodiphila, B. petrii.*

Characteristics	Strains			
	P. mirabilis	*E. coli*	*S. nematodiphila*	*B. petrii*
Shape	Bacillus	Rod	Rod	Rod to circular
Colony colour	Gray	Shiny	Bink-brown	Creamy white
Motility	+	+	+	-
Pigmentation	black-	Orange-	Red	Brown
Gram reaction	brown	red	-	-
Methyl red	-	-	-	-
Voges-proskauer	+	+	+	+
Sporulation	-	-	+	-
pH	-	-	5-9 (7.2)	7-9
Optimum temperature	4.8	6-7	5-40 (37)	35-37
Anaerobic growth	30-37	21-37	+/-	+/-
Haemolytic activity	+/-	+/-	+	-
Catalase	+	+/-	+	+
Indole production	+	+	-	-
Oxidase	-	+	-	+
Grow on :				
Sucrose	-	-	+	-
Mannose	+		-	+
d-xylose	-	+/-	+	-
l-xylose	+	+	+	-
lactose	+	+	+	-
l- sorbitol	-	+	+	-
Rhamenose	-	+	+	-
Glucose	+	+	+	-
d-arabinose	+	+	+	-
l- arabinose	-	+	-	-
Maltose	-	+	+	-
Manitol	-	+	+	+
Decomposition of:				
Starch	+	+	+	-
Gelatin	+	+	+	-
Urea	+	-	-	-
Casein	-	-	+	-
Cellulose	+	-	+	-
Tryptophan	-	+	+	-
Arginine	+	+	+	-
Lysine	-	+/-	+	-
Ornithine	+	+	+	-
Nitrogen reduction	+	+/-	-	-
Phenylalanine deaminase	+	-	-	-
H_2S	+	-	-	-

Molecular identification

Using PAUP program (Swofford, 2003), the aligned 1422 nucleotides among the different bacterial taxa and their related species showed 1074 constant sites and 348 variable sites. Among the variable nucleotides, 314 were parsimony-informative and 34 were uninformative under the parsimony criteria. The data exhibited consistency index (CI = 0.85), homology index (HI = 0.15), retention index (RI = 0.944) and rescaled consistency index (RC = 0.803). Excluding the uninformative characters, both consistency and homology indices showed values of 0.845 and 0.154, respectively. The base composition for the studied strains were A= 24.95%, C= 23.39%, G= 31.57% and T= 20.09%. The ML analysis for the data was conducted by the nearest-neighbour interchange (NNI) exhibiting a negative log likelihood score -lnL = 4048.80 and Akaike Information Criterion AIC= 8113.61. The best-fit model that explained the datasets was GTR+G+I showing model parameters as follows: substitution rate matrix R (a) =1.00; R (b) = 4.08; R (c) = 1.655; R (d) = 1.655, R (e) = 7.03 and R(f)= 1.00. Among-site rate variation showed the proportion of invariable sites (I) to be 0.575 and gamma distribution shape parameter to be 0.343.

A single neighbour-joining tree was shown and the bootstrap supports at the different nodes were calculated by 10000 replications for MP and NJ and by 200 replications for the ML (Fig. 1). The 8 studied strains were identified into 4 different non congeneric species which were *Proteus mirabilis* (S60, S61, S63 and S68), *Bordetella petrii* (S64) *Serratia. nematodiphila* (S66 and S67) and *Escherichia coli* (S62). Each of these well-identified strains clustered within its congeneric species with 100 % bootstrap supports. However, S62 was identified as *Escherichia coli* by NJ method only (96 % bootstrap support).

Table 4. Antibiotics susceptibility of selected strains: *P. mirabilis, E. coli, S. nematodiphila, B. petrii.*

Antibiotics	**Strains**			
	P. mirabilis	***E. coli***	***S. nematodiphila***	***B. petrii***
Ampicillin	+	-	+	+
Cefotaxime	+	-	+	+
Levoflxacin	+	+	-	+
Chloramphenicol	+	+	+	+
Flucloxacillin	-	+	+	-
Tobramycin	+	+	+	+
Tetracycline	+	+	+	+
Ofloxacin	+	+	-	+
Norfloxacin	+	+	+	+
Imipenem	+	+	-	+

\+ Inhibit the bacterial growth -Resistant to the bacterial growth

Table 5. Pairwise genetic distances calculated from the 16S rDNA data for the *different bacterial* strains studied.

	S60	***P. mirabilis***	**S64**	***B. petrii***	**S62**	***E. coli***	***S. flexneri***	**S66**	***S. nematodiphila***
S60, S61,S63, S68	-								
P. mirabilis	0.000	-							
P. vulgaris	0.009	0.009							
S64			-						
Bordetella petrii			0.004	-					
Bordetella avium			0.010	0.011					
S62					-				
Escherichia coli					0.003	-			
Shigella flexneri					0.006	0.009	-		
S66, S67								-	
Serratia nematodiphila								0.000	-
Serratia marcescens								0.001	0.001

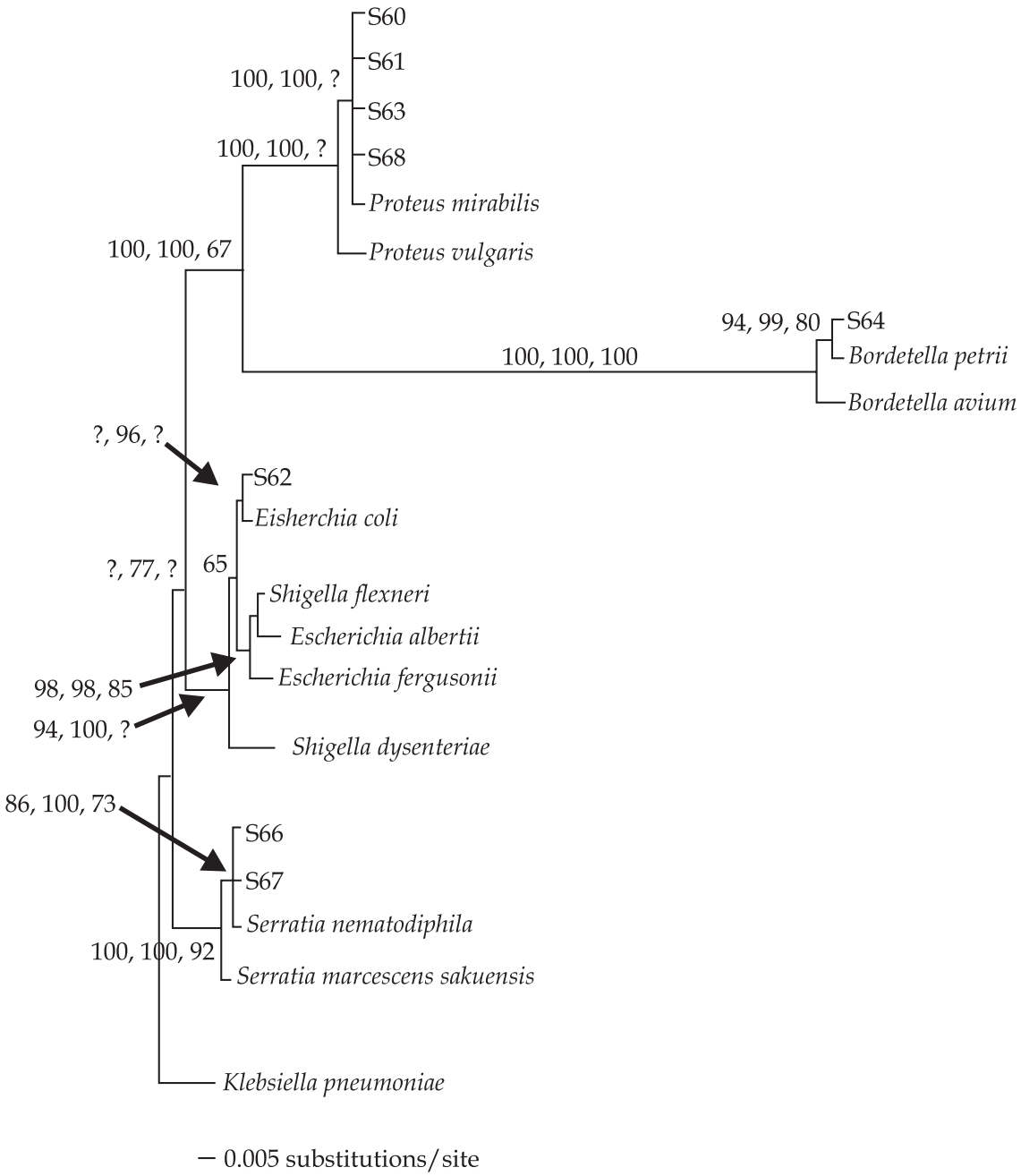

Fig 1. Neighbour-joining tree constructed from 1422 bp of 16S rDNA gene for 8 bacterial strains belonging to different 4 genera. Values at nodes represent the bootstrap support for MP, NJ and ML methods, respectively. (?) refers to that the bootstrap values are not given for the corresponding analytical method.

The aligned data were used to estimate the pairwise genetic distances among the studied strains (Table 5). In accordance to tree topology, the genetic distance showed identity between S60 and *P. mirabilis* (D= 0.000) and between S66 and *S. nematodiphila* (D= 0.000). With respect to the other strains, the smallest genetic distances were recorded between S62 and *E. coli* (D= 0.003) and between S64 and *B. petrii* (D=0.004). The smallest genetic distance exhibited between S62 and *E. coli* supports the clustering of both taxa in the tree topology. The molecular data clearly discriminated among the different strains and the tree topology is concordant with the biochemical data in constructing the relationship among the studied taxa.

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