OPEN ACCESS

African Journal of **Microbiology Research**

7 March 2018
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

AcademicJournals

Academic Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.me/

Editors

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology Research

Table of Contents: Volume 12 Number 9 7 March, 2018

ARTICLES

academicJournals

Vol. 12(9), pp. 200-217, 7 March, 2018
DOI: 10.5897/AJMR2017.8756
Article Number: C53679356215
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* L. Walp) growing in Ethiopia are nodulated by diverse rhizobia

Tulu Degefu[1*], Endalkachew Wolde-meskel[2], Zikie Ataro[3], Asnake Fikre[1], Tilahun Amede[1] and Chris Ojiewo[1]

[1]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT-Ethiopia), Addis-Ababa, Ethiopia.
[2]International Livestock Research Institute, P. O. Box 5689, Addis Ababa, Ethiopia.
[3]Department of Biology, College of Natural and Computational Sciences, Hawassa University, P. O. Box 05, Hawassa, Ethiopia.

Received 2 November, 2017; Accepted 14 December, 2017

A total of eighty one (81) rhizobial isolates were recovered from root nodules of cowpea (*Vigna unguiculata* L. Walp.) and groundnut (*Arachis hypogaea* L.) grown in soils collected from eight different sites (Hawassa, Wondogenet, Chofa, Badawacho, Bodity, Gofa, Ziway, and Alemtena) in Ethiopia with no known history of inoculation. The test isolates together with seven reference strains belonging to five genera including *Rhizobium, Ensifer, Mesorhizobium, Bradyrhizobium* and *Azorhizobium* were characterized using ninety phenotypic traits. Thirty one isolates (38%) were found to be fast growers while fifty isolates (62%) were slow growers. The majority of the isolates showed an intrinsic resistance to antibiotics (µg/ml), Chloramphenicol (5 and 15), Lincomycin (100), Novobiocin (0.5 and 1.5), and Erythromycin (10 and 20) and to heavy metals manganese sulphate (500) and copper chloride (100). Most isolates did not tolerate NaCl concentration >3% (w/v) and high temperature (45°C). Dendrogram was constructed by applying the unweighted pair group method with arithmetic means (UPGMA) using NTSYSpc Version 2.1. They were grouped into seven clusters and eight unclustered positions, when 82% relative similarity was used as a cut point. Fifty eight percent of the test isolates were grouped with *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* superclades, thus indicating that rhizobia nodulating cowpea and groundnut are delineated within a branch that defines *Bradyrhizobium* genus. To elucidate the precise taxonomic positions of the isolates, further genetic studies are required using modern molecular biological methods.

Key words: Groundnut, cowpea, isolates, phenotypic traits, *Bradyrhizobium, Rhizobium*.

INTRODUCTION

Grain legumes such as groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* L. Walp.) are essential food sources in tropical and sub-tropical regions, including Ethiopia (Duke, 2012; Singh et al., 2003; Steele, 1985). Specifically, they sustain the nutritional balances of low income societies (Zhang et al., 2007). Grain legumes have a substantial dietary value for humans and animals due to their high contents in

proteins, vitamins and minerals (Ahenkora et al., 1998; Giller, 2001; Hallensleben et al., 2009; Singh et al., 2003). Besides their nutritional qualities, legumes with their biomass are known to improve soil fertility (Senaratne et al., 1995), a desirable feature in low input smallholder agriculture.

Generally, these benefits refer to the ability of legumes to establish, in their root zone, a symbiosis with rhizobia to initialize a process defined as biological nitrogen fixation (BNF) (Boogerd and van Rossum, 1997; Dakora, 2000; Dakora and Keya, 1997). In this respect, groundnuts and cowpea have been studied extensively with respect to their BNF capability and soil fertility benefits (Senaratne et al., 1995). For example, groundnut and cowpea revealed a net contribution of N to soil up to 100 kg N ha^{-1} (Toomsan et al., 1995) and 150 kg ha^{-1} (Dakora et al., 1987), respectively when they are associated with N-fixing soil bacteria generally known as rhizobia. For these reasons, subsistence farmers in sub-Saharan Africa usually intercrop their cowpea with maize, sorghum, millet, and cassava (Langyintuo et al., 2003). In Ethiopia, cowpea is mainly grown in the drier regions (Hararge, Konso) (Westphal, 1974), and it is getting importance from time to time. With respect to nutrition, over 90% of the world groundnut is produced in developing countries and roughly two-thirds of this is used for oil, making it the most important source of vegetable oil next to soybean (Giller, 2001). Groundnut is also the third most important vegetable protein, while cowpea is used for human food, as concentrate for farm animals, hay, silage, pasture, soil cover, and green manure (Westphal, 1974). In Ethiopia, groundnut is cultivated in eastern Hararghe (Babile area), which is the peanut belt of the country and western and southern part of the country (Pawe, Gojam, Illubabour, Gamo Gofa, Welega) are also potential groundnut producing areas (Wakjira, 1992).

N-fixing rhizobia are of polyphyletic origin that spread over various taxonomic groups within the different sub-classes of alpha-, beta- and gamma-proteobacteria. Notably, those rhizobia that require a match with individual legume counterparts to induce BNF are affiliated to alpha-proteobacteria. These include the genera *Allorhizobium (*de Lajudie et al., 1998), *Azorhizobium* (Dreyfus et al., 1988), *Rhizobium* (Jordan, 1984), *Mesorhizobium* (Jarvis et al., 1997), *Ensifer* (formerly *Sinorhizobium*) (de Lajudie et al., 1994) and *Bradyrhizobium* (Jordan, 1982).

The genus *Bradyrhizobium*, however, was created to circumscribe those N-fixing bacteria that establish a symbiosis with a variety of legumes including cowpea and groundnuts and distributed broadly over tropical and temperate regions. It is acknowledged that the BNF efficacy varies depending on the combination of the legume variety and N-fixing microsymbiont strain, climatic and edaphic conditions (Giller et al., 2013). In Ethiopia, there is limited information on indigenous rhizobia that nodulate cultivated legume crops including cowpea and groundnut in various locations in the country. Few studies available on Ethiopian collections, however, showed that Ethiopian soils harbor diverse populations of rhizobia with distinct genomic composition (Beyene et al., 2004; Degefu et al., 2013; Degefu et al., 2017; Wolde-Meskel et al., 2005). Furthermore, recently, we reported several phenotypic clusters of rhizobia from nodules of chickpea and pigeon pea growing in Ethiopia (Degefu et al., 2018; Negash et al., 2018). Hence, there is strong reason to believe that there is a large, uncovered biodiversity among rhizobial population nodulating different legumes including cowpea and groundnut in Ethiopia, an acknowledged geographic centre of many leguminous plants (Table S1) (Lie et al., 1987). Furthermore, to exploit the potential benefit from BNF and to improve the agricultural productivity, it is desirable to characterize the indigenous population of rhizobia compatible to cultivated crops and develop broad host range inoculants for use in various locations. This fact necessitates the need investigating the diversity of rhizobia isolated from cowpea and groundnut extensively cultivated in Ethiopia. Hence, eighty one rhizobial strains isolated from root nodules of the two target legumes species grown at diverse locations in Ethiopia were characterized using numerical taxonomic approach. The morphophysiological diversity of the test isolatesand their relative placement, on the dendogram, with respect to the seven reference strains included in our study were exploited by analyzing ninety phenotypic traits. Furthermore, preliminary symbiotic effectiveness test (based on leaf and nodule colors) were determined.

MATERIALS AND METHODS

Isolating rhizobia from nodules

Rhizobia were isolated from nodules collected from Hawassa, Chofa, Wondo Genet, Badawacho, Bodity, Gofa, Ziway, and Alem-Tena. The nodules were preserved *in silica* gel or from fresh nodules following the methods detailed elsewhere (Somasegaran and Hoben, 1994). Desiccated nodules were imbibed in sterile water overnight. Then the nodules were surface sterilized in 95% ethanol for 5 to 10 s and 3% solution of sodium hypochlorite (NaOCl) for 2 to 4 min, rinsed in five changes of sterile distilled water on sterilized Petri dishes using flame-sterilized blunt-tipped pair of forceps. The surface sterilized nodules were crushed in drops of sterile distilled water and a loopful of the crushed nodules sap was streaked on a Yeast Extract Mannitol Agar (YEMA) plate. The plate were incubated at 28±2°C and observed for growth daily. A single colony typical of rhizobia from primary isolation plates were

*Corresponding author. E-mail: tuludege@gmail.com. Tel: +251927996699.

Table 1. Leaf color, nodule color, nodule number and nodule dry weight per plant.

Leaf color		Nodule color	Nodule No./plant min-max/average	Nodule dry wt./plant min-max/average (mg)
Deep green	GN	28 deep red	10-135/60	10-95/44.7
	CP	22 pink	15-30/23	15-87/43.5
Green	GN	7 pink	5-90/35	10-50/25.7
	CP	4 pink	19-56/34	25-27/25.5
Pale green	GN	5 white	6-22/11	7.5-10/8.4
	CP	4 white	8-38/19	7.5-10/9.4
Yellow	GN	11 white	*	*

GN: Groundnut, CP: cowpea, *Many in number but very small in size.

re-isolated by streaking on YEMA containing (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.5 g, and 15 g agar in 1 L distilled water) containing bromothymol blue (BTB) as pH reaction indicator, YEMA containing congo red (CR) and peptone glucose agar (PGA) (glucose 5 g, peptone 10 g, agar 15 g and 10 ml BCP stock solution per liter distilled water) containing bromocresol purple (BCP) and gram-stained as a presumptive test (Odee et al., 1997).

Authentication of the isolates as rhizobia

A rhizobial strain was tested for its ability to produce nodules on homologous host. Seedlings were grown in modified Leonard jar constructed from plastic pots which were filled with sterilized river sand. Surface sterilized and pregerminated seeds were transplanted into the pots. Each strain of rhizobia isolated was grown in yeast extract mannitol broth (YEMB) to logarithmic phase and the seedlings were inoculated with 1 ml of each isolates. The extra growth units which were not inoculated served as controls, negative control (non-inoculated and supplied with N-free nutrient solution). The plants were grown in triplicate under greenhouse condition. The pots were arranged in completely randomized design (CRD) and fertilized with quarter-strength modified Jensen's N-free medium ($CaHPO_4$ 1 g, K_2HPO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.2 g, trace elements stock solution 1 ml and $FeCl_3$ 0.1 g/L of distilled water). All seedlings were checked for nodulation after 45 days (Maâtallah et al., 2002; Somasegaran and Hoben, 1994). Nodule number per pot and internal color were scored. Nodules were dried at 80°C for 24 h and their dry weights were measured.

Characterization of the isolates

Morphological characteristics

After incubation of 3 to 13 days at 28°C, the colony morphology (Odee et al., 1997; Somasegaran and Hoben, 1994) and acid base production (Jordan, 1984) were examined following the procedures detailed in the respective references.

Biochemical and physiological characteristics

Sodium chloride (NaCl) tolerance (with concentration of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5% (w/v)) (Amarger et al., 1997), temperature (5, 20, 25, 30, 40 and 45°C), tolerance (Maâtallah et al., 2002), pH (4, 4.5, 5, 5.5, 6, 7, 8, 8.5, 9, 9.5, 10, and 10.5) (Amarger et al., 1997), utilization of different carbon sources including arabinose, rhamnose, xylose, galactose, mannose, maltose, trehalose, dextrin, inulin, raffinose, dulcitol, sorbitol, citric acid, malonic acid, xylitol, myo-inositol, and ribose (Somasegaran and Hoben, 1994), utilization of different nitrogen sources (L-aspargine, L-proline, L-leucine, L-alanine, L-theronine, L-arginine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-histidine, Inosine, uredine, thymidine, glutamic acid, aspartic acid, and glycine) (Amarger et al., 1997), the resistance of the test isolates to different antibiotic (novobiocin (0.5, 1.5), streptomycin (10), spectinomycin (2.5, 5), kanamycin (5, 15), erythromycin (10, 20), chloramphenicol (5, 15), neomycin (5, 20), trimethoprim (200), and lincomycin (100), heavy metals ($MnSO_4.H_2O$, 500 µg/ml; Pb $(CH_3COOH).3H_2O$, 500 µg/ml; $ZnCl_2$, 100 µg/ml; CuCl, 100 µg/ml; $CoCl_2.6H_2O$, 500 µg/ml; and $AlCl_3.6H_2O$, 500 µg/ml) (Zhang et al., 1991) and phosphate solubilizing ability (Alikhani et al., 2006) were determined following the methods and procedures described in the respective references.

Numerical analysis

To investigate the phenotypic variability among the isolates, a dendrogram was constructed by using the average Unweighted Pair Group Method with Arithmetic Means (UPGMAM) using NTSYSpc version 2.1. Characters were coded as 1 for positive (growth) and 0 for negative (no growth).

RESULTS AND DISCUSSION

In total, 81 rhizobial isolates were isolated from root nodules of groundnut (51 isolates) and cowpea (30 isolates) (Table S1). Presumptive tests indicated, all the 81 test isolates were found to be Gram negative, formed white colonies on YEMA containing CR and showed no growth on PGA containing BCP, which are a characteristics feature of rhizobia (Somasegaran and Hoben, 1994). All isolates were able to nodulate their host species confirming that they are rhizobia. Preliminary symbiotic effectiveness test indicated that sixty one strains (35 from groundnuts and 26 from cowpea) formed effective nodule with deep green/green leaf color while twenty strains (16 from groundnut and 4 from cowpea) formed ineffective nodules with pale green/yellow leaf color (Table 1).

Table 2. Colony characteristics, acid and base production and date of first colony appearance.

Property		Cowpea	Groundnut	Total no. of isolates	%
Colony color	WT	21	40	61	75
	Milky	9	11	20	25
Colony size	<1 mm	25	25	50	62
	1-2 mm	4	18	22	27
	>2 mm	1	8	9	11
Shape	Convex	30	48	78	96
	Flat	-	3	3	4
Texture	Buttery	30	51	81	100
EPS production	-	-	10	10	12
Acid reaction	-	5	23	28	35
Alkaline r×n	-	25	28	53	65
Date 1st colony appeared	3-5 days	8	23	31	38
	5-10 days	22	28	50	62

WT: Watery translucent.

Colony characteristics

The test isolates varied in colony size; 62% of them formed small colonies with diameter of <1 mm in 5 to 10 days. This is in agreement with other similar work, where 67% of the strains formed colonies with diameter of ≤1 mm (Hungria et al., 2001). On the other hand, 27% formed colonies with diameter ranging between 1 and 2 mm when incubated for 4 to 5 days, and 11% of the test isolates formed larger colonies (2 to 4.2 mm in diameter) upon incubating them for 3 to 4 days. It has been reported that fast-growers form visible colonies on YEMA within 3 to 5 days and slow-growers, however, need more than 5 days to form colonies with diameter of 1 mm under the same conditions (Jordan, 1984). Accordingly, thirty one test strains (38%) were found to be fast growers while fifty test strains (62%) were slow growers. Convex elevation was detected in 96% of the isolates while only 4% (3 groundnut strains GZ014, GZ018 and GG060) were found to appear with colony shape as flat. The majority of the isolates (75%) expressed watery translucent colony color and the remaining 25% were milky. Only 12% of the isolates, all from groundnut, produced extracellular polysaccharide (EPS) (Table 2).

Reaction on YMA-BTB

Twenty eight isolates (35%) were acid producers and changed the media to yellow when incubated for 5 days on YEMA-BTB media. Fifty three isolates (65%) had alkaline reaction in which the media were changed to blue. Slow growing rhizobia produce alkaline reaction on YEMA-BTB while fast growing rhizobia produce acid (Jordan, 1984). However, unlike the fast-growing ones which commonly produce acid reaction, three isolates from cowpea (CC027, CC028 and CC029) were fast growers but alkaline producers. This indicates that reaction of rhizobia on YEMA-BTB media cannot be considered as diagnostic feature. Similar results have been reported in earlier undertakings of similar nature (Moreira et al., 1993; Wolde-Meskel et al., 2004a, b).

pH and temperature tolerance of the isolates

With the respect to pH profiles, all isolates grew at pH values ranging between 5.5 and 10.5 but 77% of cowpea and 69% of groundnut test strains grew at pH value as low as 4.0. On the other hand, 97% of cowpea and 90% of groundnut strains grew at pH 4.5 while 97% of the isolates from cowpea and 96% of the isolates from groundnut were able to grow at pH 5.0 (Table 3). In summary, all the tested isolates were found to be tolerant to high pHs. In earlier studies on rhizobia from cowpea and mungbean (Zhang et al., 2006) and chickpea (Nour et al., 1994), it was reported that rhizobial strains could grow at pH values ranging between 5.0 and 11.0 (for cowpea and mungbean isolates) and as high as 10.0 (for chickpea isolates). The isolates, which were found to have grown in a wider pH ranges, may have practical application with respect to selection of a wide-range pH tolerant strains that can perform well under acidic, neutral and alkaline soils.

Table 3. pH and temperature tolerance of the test isolates.

Site	No. of isolates	Proportion of tolerant isolates (%) at							
		pH levels				Temperature (°C)			
		4.0	4.5	5.0	5.5-10.5	5	20-30	40	45
Hawassa	CP 7	85	85	100	100	-	100	-	-
	GN 19	63	73	100	100	5	100	11	-
Ziway	CP 4	75	100	100	100	-	100	-	-
	GN 9	11	11	78	100	-	100	100	-
Bodity	CP 5	100	100	100	100	-	100	-	-
	GN 1	100	100	100	100	-	100	-	-
Gofa	GN 21	86	90	100	100	5	100	76	-
Badawacho	GN 1	100	100	100	100	-	100	100	-
Chofa	CP 10	40	60	90	100	-	100	10	-
Wondogenet	CP 2	100	100	100	100	-	100	50	50
Alemtena	CP 2	-	-	100	100	-	100	50	-

Regarding temperature requirements, all the rhizobial isolates in this study were able to grow at temperature values ranging between 20 and 30°C (Table 3), which to some extent was in agreement with others that reported the optimum growth temperature range for rhizobia varies between 25 and 31°C (Jordan, 1984; Somasegaran and Hoben, 1994). Only two isolates from groundnut (GG055 and GH101) were found to be tolerant to low temperature (5°C). About 7% of isolates from cowpea and 53% of isolates from groundnuts were able to grow at 40°C. One isolate (GH026) from groundnut tolerated a temperature of 45°C.

Similar to the present finding, the ability of rhizobial isolates to grow at high temperature were also reported by others (Zahran et al., 1994; Zhang et al., 1991). Practically, the existence of the isolates that could tolerate high temperature could potentially be helpful to develop inoculant that can perform better at high temperature, since cowpea and groundnuts grow in low lands where day temperature can be higher.

Salt tolerance

Tolerance of the test isolates to various salt (NaCl) concentrations is presented in Table 4. Accordingly, all the tested rhizobial isolates exhibited a wide range of variations in their tolerance to NaCl concentration. As the concentration of NaCl was increased the growth of the isolates were found to be inhibited. All the 81 rhizobial isolates grew in YEMA with 0.5 and 1% NaCl. But the proportion rapidly decreased as the concentration of NaCl increased. Thus, for cowpea, the tolerant isolates decreased from 83 to 3% when the NaCl concentration increased from 1.5 to 5.5%. For groundnut isolates, the proportion reduced from 80 to 2% when the NaCl concentration increased from 1.5 to 5.5%. Five rhizobial isolates, three from groundnut (GH026, GG055 and GH101) and two from cowpea (CC23 and CZ42) were found to be tolerant to a salt concentration of 4.5%. Only three isolates, two from groundnut (GH026, GH101) and one from cowpea (CC23) were found to be tolerant to a salt concentration of 5.5%.

It was reported that rhizobia can generally grow at salt concentration as high as 2% (Jordan, 1984). Another study on isolates from chickpea indicated that 3 out of 56 isolates were tolerant to 5% NaCl concentration (Maâtallah et al., 2002). The presence of such salt tolerant isolates in the collection can be regarded as a resource for applied research aiming to develop inoculant for the target legumes growing at localities where salinity is a problem.

Carbon and nitrogen source utilization

The ability of the test isolates to utilize different substrates as sole carbon and nitrogen source (Table S2) were presented. All of the tested isolates were able to grow on trehalose, raffinose, maltose, sorbitol, arabinose, xylitol, sucrose, mannitol, myo-inositol, D-glucose, inulin, galactose, ribose, mannose, starch, xylose, dextrin, rhaminose, and dulcitol. However, all the tested isolates failed to grow in the presence of fructose, malonic acid and citric acid. All the isolates grew on L-proline, L-arginine, L-alanine, L-leucine, L-phenylalanine, L-glutamic acid, L-histidine, aspartic acid, inosine, uredine, L-threonine, L-asparginine, thymidine and L-

Table 4. Salt tolerance of cowpea and groundnut isolates.

Site	No. of isolates	Proportion of tolerant isolates (%) at salt concentration % (w/v)									
		0.5-1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5
Hawassa	CP 7	100	100	100	100	100	86	-	-	-	-
	GN 19	100	68	68	68	68	16	16	11	11	5
Ziway	CP 4	100	100	50	50	50	25	25	25	-	-
	GN 9	100	78	78	78	78	78	-	-	-	-
Bodity	CP 5	100	80	20	20	20	-	-	-	-	-
	GN 1	100	100	100	100	100	-	-	-	-	-
Gofa	GN 21	100	90	86	81	71	5	5	5	-	-
Badawacho	GN 1	100	100	100	-	-	-	-	-	-	-
Chofa	CP 10	100	70	40	20	20	10	10	10	10	10
Wondogenet	CP 2	100	100	100	100	100	-	-	-	-	-
Alemtena	CP 2	100	50	50	50	50	-	-	-	-	-

phyroglutamic acid as nitrogen sources. However, the following nitrogen sources including glycine and D-Serine were found to selectively inhibit the growth of quite a number of our test isolates. Accordingly, glycine and D-serine were utilized only by 31 and 30% of the isolates, respectively. The results on carbon and nitrogen utilization by cowpea and groundnut isolates in the present study agrees with other works (Amarger et al., 1997; Gao et al., 1994; Lindström and Lehtomäki, 1988; Zhang et al., 1991). In general, the results imply that the test isolates, which were able to grow on diverse carbon and nitrogen sources, have selective advantage over those grown on restricted carbon and nitrogen sources.

Intrinsic antibiotic resistance (IAR)

The ability of the test isolates, when incubated on YEMA supplemented with different antibiotics, is presented in Table 5. Isolates generally displayed resistance to chloramphenicol, lincomycin, novobiocin, erythromycin and trimethoprim but were susceptible to 15 μgml^{-1} of kanamycin, 20 μgml^{-1} of neomycin and 10 μgml^{-1} of streptomycin. The lower levels of each of the antibiotics tolerated by the majority of the isolates includes 5 μgml^{-1} of neomycin (90%), 15 μgml^{-1} of kanamycin (88%), 2.5 μgml^{-1} of spectinomycin (86%) and higher level (5 μgml^{-1}) of spectinomycin (85%). It has already been indicated that fast growing strains are more sensitive to antibiotics than slow growing ones (Jordan, 1984), which agrees with the findings. The intrinsic antibiotic resistance of the test isolates is one of the survival strategies, thus to colonize the rhizosphere.

Intrinsic heavy metal resistance

Almost all the isolates were tolerant to the tested concentration of manganese and the majority showed an intrinsic resistance to copper (96%), lead (83%) and zinc (77%). Cobalt and aluminium seemed to be selective in that 24 and 47% of the isolates, respectively, tolerated a concentration of 500 μgml^{-1} of each (Table 6). In another study of similar nature, it was found out that 67% of the strains tolerated 0.5 mM of cobalt chloride and no isolate tolerated 2.5 mM lead acetate (Hungria et al., 2001). But, in this study, most isolates (83%) tolerated lead acetate (500 μgml^{-1}). The intrinsic heavy metal resistance of the test isolates observed in this study implies that the resistant isolates could be regarded as potential candidates to develop inoculant for environments polluted with heavy metals.

Phosphate solublizing ability

Out of the 81 tested isolates, 53 (65%) were found to solubilize phosphate as confirmed by the formation of clear zone around the colonies on agar plates (Table S2). This result agrees with other results generated in Iran The results indicated that the phosphate solubilizing isolates in our collection, in addition to their N_2-fixing ability, could avail plant phosphorus nutrition by mobilizing inorganic phosphate.

Numerical analysis of phenotypic data

The result of the cluster analysis performed on the 81 test isolates and 7 reference species for 90 phenotypic characteristics is as shown in Figure 1. The result grouped the strains into 7 clusters and 8 un-clustered positions (3 isolates and 5 reference strains) using 82% similarity level as a cut point. The majority of the isolates (58%) were clustered with *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, thus indicating that the slow

Table 5. Intrinsic antibiotic resistance of the isolates.

Antibiotic	Concentration (µg/ml)	% of resistant isolates		
		Cowpea	Groundnut	Total
Chloramphenicol	5	100	100	100
	15	100	100	100
Lincomycin	100	100	100	100
Novobiocin	0.5	100	100	100
	1.5	97	100	99
Erythromycin	10	97	100	99
	20	93	100	98
Trimethoprim	500	100	92	95
Neomycin	5	97	86	90
	20	90	69	77
Spectinomycin	2.5	83	88	86
	5	83	86	85
Kanamycin	5	90	86	88
	15	73	75	74
Streptomycin	10	83	75	7 8

Table 6. Intrinsic heavy metal resistance of the isolates.

Heavy metal	Concentration (µg/ml)	% of resistant isolates		
		Cowpea	Groundnut	Total
Mn	500	97	100	99
Cu	100	97	96	96
Pb	500	73	88	83
Zn	100	83	73	77
Al	500	20	63	47
Co	500	37	16	24

growing isolates nodulating cowpea and groundnut are most likely to comprise *B. japonicum, B. elkanii*, and other unidentified *Bradyrhizobium* species. The results, in some respects, were in agreement with other work on rhizobia from *A. hypogaea* L grown on Argentinean soils (Taurian et al., 2006).

Cluster I comprised three isolates (one from cowpea grown in Alem-Tena soil and two from groundnut grown in Gofaand Hawassa soil). They were slow growers. The isolates under this cluster were able to grow at 3% NaCl concentration, failed to utilize D-serine and glycine as sole nitrogen source, sensitive to antibiotics (spectinomycin, streptomycin and kanamycin) and heavy metals including cobalt and zinc.

Cluster II consists of 47 isolates (22 from cowpea and 25 from groundnut) from six study sites (Hawassa, Wondo Genet, Chofa, Bodity, Gofa and Ziway) and two reference strains *B. japonicum* (HAMBI 2314^{T}) and *B. elkanii* (LMG 6164). This cluster contained 58% of the isolates and it was very heterogeneous with two large sub-clusters at a similarity level of 83%. Sub-cluster IIA containing 29 isolates and sub-cluster IIB contained 18 isolates and two reference strains (*B. japonicum* and *B. elkanii*). It has been reported that very fast, fast,

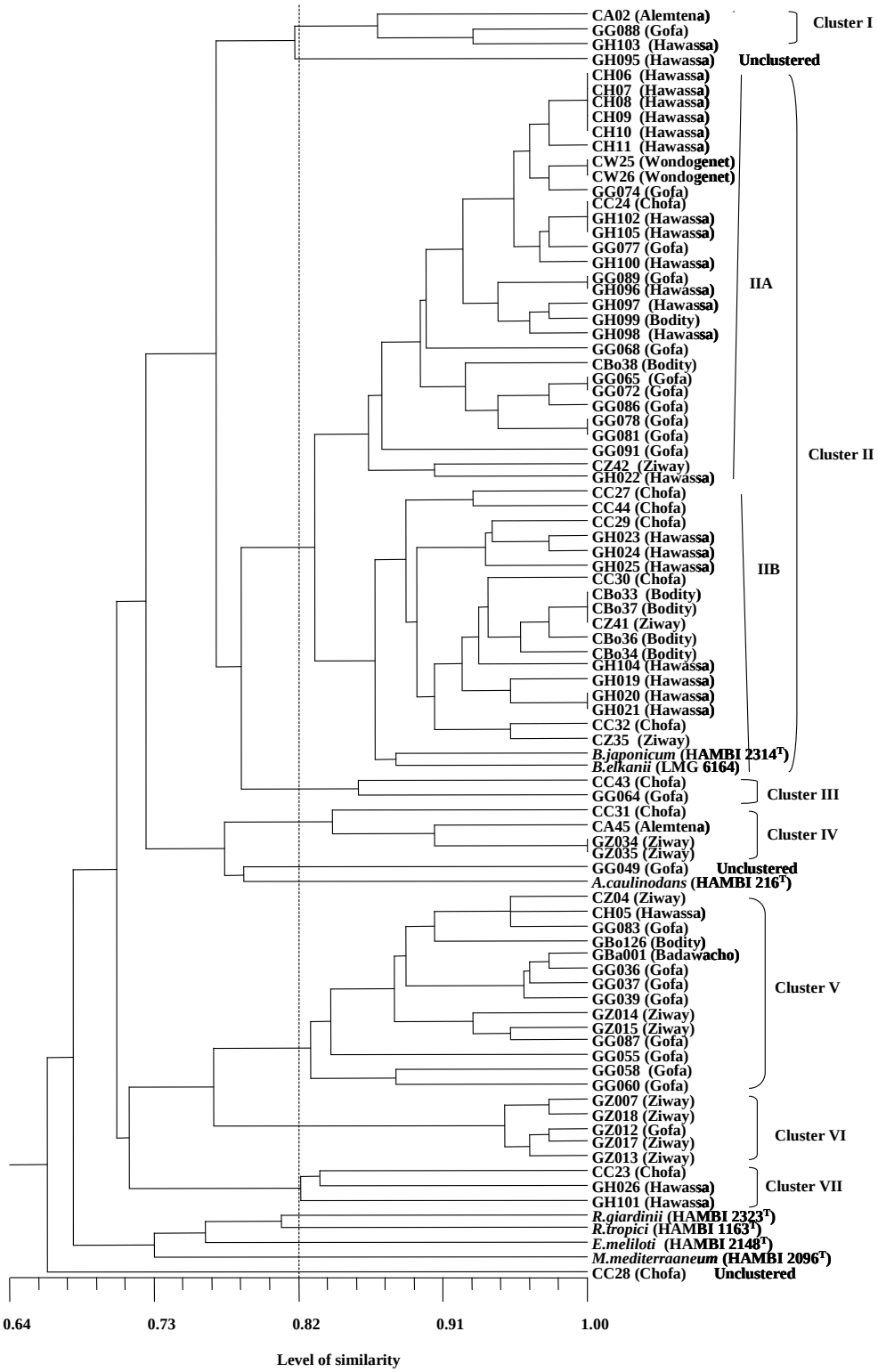


Figure 1. Dendrogram showing phenotypic similarity between test and reference strains.

intermediate and slow growing rhizobia are intermingled with one another and thus appeared as single cluster (Odee et al., 1997). Similarly, in this work slow growers and fast growers grouped together in clusters II and VII. The inclusion of *B. japonicum* and *B. elkanii* in this cluster is in agreement with other similar work, which reported

that rhizobia from cowpea grown in Senegal belong to the genus *Bradyrhizobium* (Krasova-Wade et al., 2003). On another study, rhizobia nodulating soybean and peanut growing in China were found to belong to the genus *Bradyrhizobium* (Yang and Zhou, 2008). The isolates under this cluster showed wide resistance to antibiotics, heavy metals, pH and a salt tolerance as high as 3%. They were found to utilize different substrates as their sole carbon and nitrogen sources.

Cluster III consisted of one cowpea isolate from Chofa (CC43) and one groundnut isolate from Gofa (GG064). They were fast growers. They were able to grow at pH values ranging between 4.0 and 10.5, NaCl concentration of 1%, sensitive to higher temperature, resistant to all tested antibiotics and sensitive to cobalt.

Cluster IV consisted of 4 isolates, two cowpea isolates (one from Chofa and one from Alem-Tena) and two groundnut isolates from Ziway. All the isolates under this cluster were slow growers. They were sensitive to pH 4.0, NaCl concentration of 1.5%, kanamycin, spectinomycin, streptomycin, lead, zinc, aluminum and cobalt.

Cluster V consisted of 14 isolates: two cowpea isolates (one from Ziway and one from Hawassa) and 12 groundnut isolates (eight from Gofa, two from Ziway, one from Bodity, and one from Badawacho). All the isolates were fast growers, sensitive to lower pH (4.0 and 4.5), tolerated NaCl concentration of 3%, temperature of 40°C and were resistant to heavy metals except cobalt.

Cluster VI consisted of 5 groundnut isolates from Ziway. All were fast growers, tolerated pH values ranging between 5.0 and 10.5, NaCl concentration of 3.5%, temperature of 40°C and were susceptible to kanamycin, neomycin, streptomycin, zinc and cobalt. All the five isolates in this cluster do not have phosphate solublizing ability.

Cluster VII consisted of three isolates, one cowpea isolate from Chofa and two groundnut isolates from Hawassa. The typical feature of this cluster was that they tolerated all salt concentrations tested (0.5 to 5.5%), continued to grow at a temperature of 45°C and all concentration levels of antibiotics tested.

Unclustered consisted of three isolates (GH095, GH049 and CC28) showed different test results, when compared with each other and to the other isolates in different clusters. GH095 and GH049 were isolated from groundnut grown in Hawassa soils and CC28 was isolated from cowpea grown in Chofa soil. All the three isolates tolerated pH 4.0 to 10.5, unable to utilize D-serine and glycine as nitrogen sources. But they showed wide variability in salt tolerance, IAR and intrinsic heavy metal resistance. Isolate GH095 tolerated NaCl concentration of 3% while CC28 tolerated 2% and GH049 1%. With respect to IAR, isolate CC28 was found to be susceptible to tested concentrations of kanamycin, erythromycin, spectinomycin, streptomycin and 20 μgml^{-1} of neomycin; isolate GH095 was sensitive to tested concentrations of spectinomycin, streptomycin, trimethoprim and 20 μgml^{-1} isolates of neomycin; GH049 was sensitive to tested concentrations of kanamycin, streptomycin neomycin and 5 μgml^{-1} of spectinomycin. Isolate GH049 was found to be sensitive to zinc and cobalt while GH095 was found to be sensitive to aluminium and CC28 to cobalt.

In earlier studies, based on numerical taxonomic approach, it was found that *B. japonicum* strains always clustered together with other *Bradyrhizobium* spp. at or above the 70% similarity level (Van Rossum et al., 1994). Furthermore, slowly growing rhizobia obtained from Hainan Province, China and three representative strains of *B. japonicum*, clustered together at similarity level of 81% (Gao et al., 1994). Similarly, majority of isolates (58%) under this study are clustered with *B. japonicum* and *B. elkanii* at similarity level of 82%, thus our slow growing strains might be closely related to these *Bradyrhizobium* reference strains. However, these remained to be established on further genetic analysis of the strains.

Conclusion

Based on the results generated from the authentication and preliminary symbiotic effectiveness test, it can be concluded that isolates with deep green and green leaf color and branching shoots and deep red and pink nodule color are effective in fixing atmospheric nitrogen. Morphophysiological characteristics and the clustering of the test isolates based on the 90 characters indicate that there is a wide diversity in rhizobial isolates nodulating cowpea and groundnut in the study sites. The rhizobial isolates that tolerated extreme environmental conditions may be potential candidates to develop them into inoculant for soils having such constraints. The ability of the test isolates to utilize a wide range of carbon and nitrogen sources imply that isolates with such ability can easily colonize the soil environment and compete with other microorganisms. To further elucidate the exact phylogenetic positions of the test isolates, genetic studies are required using modern molecular biological methods.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ahenkora K, Adu-Dapaah HK, Asafo-Adjei B, Asafu-Agyei JN, Adjei J, Konadu EYO (1998). Protein productivity and economic feasibility of dual-purpose cowpea. Hort. Sci. 33:1160-1162.

Alikhani HA, Saleh-Rastin N, Antoun H (2006). Phosphate solubilization activity of rhizobia native to Iranian soils. Plant Soil. 287:35-41.

Amarger N, Macheret V, Laguerre G (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Evol. Microbiol. 47:996-1006.

Beyene D, Kassa S, Ampy F, Asseffa A, Gebremedhin T, Van Berkum

P (2004). Ethiopian soils harbor natural populations of rhizobia that form symbioses with common bean (*Phaseolus vulgaris* L.). Arch. Microbiol. 181:129-136.
Boogerd FC, van Rossum D (1997). Nodulation of groundnut by *Bradyrhizobium*: A simple infection process by crack entry. FEMS. Microbiol. Rev. 21: 5-27.
Dakora FD (2000). Commonality of root nodulation signals and nitrogen assimilation in tropical grain legumes belonging to the tribe Phaseoleae. Funct. Plant. Biol. 27:885-892.
Dakora FD, Aboyinga RA, Mahama Y, Apaseku J (1987). Assessment of N2 fixation in groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* L. Walp) and their relative N contribution to a succeeding maize crop in Northern Ghana. J. Appl. Microbiol. Biotechnol. 3: 389-399.
Dakora FD, Keya SO (1997). Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. Soil. Biol. Biochem. 29: 809-817.
de Lajudie P, Laurent-Fulele E, Willems A, Torek U, Coopman R, Collins MD, Kersters K, Dreyfus B, Gillis M (1998). *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J. Syst. Evol. Microbiol. 48: 1277-1290.
de Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins MD, Dreyfus B, Kersters K, Gillis M (1994). Polyphasic Taxonomy of Rhizobia: Emendation of the Genus *Sinorhizobium* and Description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Evol. Microbiol. 44:715-733.
Degefu T, Wolde-Meskel E, Liu B, Cleenwerck I, Willems A, Frostegard A (2013). *Mesorhizobium shonense* sp. nov., *Mesorhizobium hawassense* sp. nov. and *Mesorhizobium abyssinicae* sp. nov., isolated from root nodules of different agroforestry legume trees. Int. J. Syst. Evol. Microbiol. 63:1746-53.
Degefu T, Wolde-meskel E, Rasche F (2017). Genetic diversity and symbiotic effectiveness of *Bradyrhizobium* strains nodulating selected annual grain legumes growing in Ethiopia. Int. J. Syst. Evol. Microbiol. DOI 10.1099/ijsem.0.002486
Degefu T, Wolde-meskel E, Makka A, Fikre A, Amede T, Ojiewo C (2018). Morphophysiological diversity of rhizobia nodulating pigeon pea (*Cajanus cajan* L. Millsp.) growing in Ethiopia. Afr. J. Biotechnol. 17: 167-177.
Dreyfus B, Garcia JL, Gillis M (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Evol. Microbiol. 38: 89-98.
Duke J (2012). Handbook of legumes of world economic importance," Springer Science & Business Media.
Gao JL, Sun, JG, Li Y, Wang ET, Chen WX (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. Int. J. Syst. Evol. Microbiol. 44:151-158.
Giller KE (2001). "Nitrogen fixation in tropical cropping systems," Cabi, London.
Giller KE, Franke AC, Abaidoo R, Baijukya F, Bala A, Boahen S, Dashiell K, Kantengwa S, Sanginga JM, Sanginga N, Simmons AJ, Turner A, de Wolf J, Woomer P, Vanlauwe B (2013). N2Africa: Putting nitrogen fixation to work for smallholder farmers in Africa. In: Vanlauwe B, van Asten PJA, Blomme G (Eds.), Agro-ecological Intensification of Agricultural Systems in the African Highlands. Routledge, London, pp. 156-174.
Hallensleben M, Polreich S, Heller J, Maass BL (2009). Assessment of the importance and utilization of cowpea (*Vigna unguiculata* L. Walp.) as leafy vegetable in small-scale farm households in Tanzania-East Africa. *http://hdl.handle.net/10568/56192*
Hungria M, de O Chueire LGM, Coca RG, Megías M (2001). Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil. Soil. Biol. Biochem. 33: 1349-1361.
Jarvis BDW, Van Berkum P, Chen WX, Nour SM, Fernandez MP, Cleyet-Marel JC, Gillis M (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri, Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. Int. J. Syst. Evol. Microbiol. 47:895-898.
Jordan DC (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a Genus of Slow-Growing, Root Nodule Bacteria from Leguminous Plants. Int. J. Syst. Bacteriol. 32:136-139.
Jordan DC (1984). Family III. *Rhizobiaceae*. Bergey's Man. Syst. Bacteriol. 1:234-242.
Krasova-Wade T, Ndoye I, Braconnier S, Sarr B, De Lajudie P, Neyra M (2003). Diversity of indigeneous bradyrhizobia associated with three cowpea cultivars (*Vigna unguiculata* (L.) Walp.) grown under limited and favorable water conditions in Senegal (West Africa). Afric. J. Biotechnol. 2:13-22.
Langyintuo AS, Lowenberg-DeBoer J, Faye M, Lambert D, Ibro G, Moussa B, Kergna A, Kushwaha S, Musa S, Ntoukam G (2003). Cowpea supply and demand in West and Central Africa. Field Crops Res. 82: 215-231.
Lie TA, Göktan D, Engin M, Pijnenborg J, Anlarsal E (1987). Co-evolution of the legume-Rhizobium association. *In* "Plant and Soil Interfaces and Interactions", Springer. pp. 171-181.
Lindström K, Lehtomäki S (1988). Metabolic properties, maximum growth temperature and phage sensitivity of *Rhizobium* sp.(*Galega*) compared with other fast-growing rhizobia. FEMS. Microbiol. Lett. 50:277-287.
Maâtallah J, Berraho EB, Sanjuan J, Lluch C (2002). Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum*) growing in Moroccan soils. Agronomy 22:321-329.
Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA (1993). Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis of their total proteins. Syst. Appl. Microbiol. 16:135-146.
Negash D, Degefu T, Alazar E, Ojiewo C (2018). Phenotypic characteristics of rhizobial and nonrhizobial isolates recovered from root nodules of chickpea (*Cicer arietinum* L.) grown in Ethiopia. Afr. J. Microbiol. Res. 12: 73-85.
Nour SM, Fernandez MP, Normand P, Cleyet-Marel JC (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). Int. J. Syst. Evol. Microbiol. 44:511-522.
Odee DW, Sutherland JM, Makatiani ET, McInroy SG, Sprent JI (1997). Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. Plant. Soil. 188: 65-75.
Senaratne R, Liyanage NDL, Soper RJ (1995). Nitrogen fixation of and N transfer from cowpea, mungbean and groundnut when intercropped with maize. Fertil. Res. 40: 41-48.
Singh BB, Ajeigbe HA, Tarawali SA, Fernandez-Rivera S, Abubakar M (2003). Improving the production and utilization of cowpea as food and fodder. Field Crops Res. 84:169-177.
Somasegaran P, Hoben HJ (1994). Collecting nodules and isolating rhizobia. *In* "Handbook for Rhizobia", Springer. pp. 7-23.
Steele WM (1985). Cowpea (*Vigna unguiculata* (L.) Walp.). Grain Legume Crops. pp. 520-583.
Taurian T, Ibañez F, Fabra A, Aguilar OM (2006). Genetic diversity of rhizobia nodulating *Arachis hypogaea* L. in central Argentinean soils. Plant Soil. 282:41-52.
Toomsan B, McDonagh JF, Limpinuntana V, Giller KE (1995). Nitrogen fixation by groundnut and soyabean and residual nitrogen benefits to rice in farmers' fields in Northeast Thailand. Plant Soil. 175:45-56.
Van Rossum D, Muyotcha A, Bram M, van Verseveld HW, Stouthamer AH, Boogerd FC (1994). Soil acidity in relation to groundnut-*Bradyrhizobium* symbiotic performance. Plant Soil. 163:165-175.
Wakjira A (1992). Oil Seeds Research and Developing in Ethiopia (Groundnut breeding). IAR. pp. 51-56.
Westphal, E. (1974). Pulses in Ethiopia, their taxonomy and agricultural significance. DOI10.2307/4103104.
Wolde-meskel E, Berg T, Peters NK, Frostegård Å (2004a). Nodulation status of native woody legumes and phenotypic characteristics of associated rhizobia in soils of southern Ethiopia. Biol. Fertil. Soils. 40: 55-66.
Wolde-Meskel E, Terefework Z, Frostegard A, Lindstrom K (2005). Genetic diversity and phylogeny of rhizobia isolated from agroforestry legume species in southern Ethiopia. Int. J. Syst. Evol. Microbiol. 55:1439-1452.
Wolde-meskel E, Terefework Z, Lindström K, Frostegård Å (2004b).

Metabolic and genomic diversity of rhizobia isolated from field standing native and exotic woody legumes in southern Ethiopia. Syst. Appl. Microbiol. 27:603-611.
Yang JK, Zhou JC (2008). Diversity, phylogeny and host specificity of soybean and peanut bradyrhizobia. Biol. Fertil. Soils. 44:843-851.
Zahran HH, Räsänen LA, Karsisto M, Lindström K (1994). Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. World. J. Microbiol. Biotechnol. 10:100-105.
Zhang WT, Yang JK, Yuan TY, Zhou JC (2007). Genetic diversity and phylogeny of indigenous rhizobia from cowpea [*Vigna unguiculata* (L.) Walp.]. Biol. Fertil. Soils. 44:201-210.
Zhang X, Harper R, Karsisto M, Lindström K (1991). Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. Int. J. Syst. Evol. Microbiol. 41:104-113.
Zhang YF, Wang FQ, Chen WX (2006). Numerical taxonomy and 16S rDNA PCR-rFLP analysis of rhizobial strains isolated from root nodules of cowpea and mung bean grown in different regions of China. Acta. Microbiol. Sinica. 46: 861-868.

Table S1. Strain designations, geographic location and trap host species from which the test strains were isolated.

Location	Strain designation		No of isolates		
	Groundnut	Cowpea	Gn	Cp	T
Hawassa	GH019, GH020, GH021, GH022, GH023, GH024, GH025, GH026, GH095, GH096, GH097, GH098,GH099, GH100, GH101, GH102,GH103, GH104, GH105	CH05, CH06, CH07, CH08, CH09, CH10, CH11	19	7	26
Ziway	GZ007, GZ012, GZ013, GZ014, GZ015, GZ017, GZ018, GZ034, GZ035	CZ04, CZ35, CZ41, CZ42	9	4	13
Bodity	GBo126	CBo33, CBo34,CBo36, CBo37, CBo38	1	5	6
Gofa	GG036, GG037, GG039,GG049, GG055, GG058, GG060, GG064, GG065, GG068, GG072, GG074, GG077, GG078, GG081, GG083, GG086, GG087, GG088, GG089, GG091	-	21	-	21
Badawacho	GBa001	-	1	-	1
Chofa	-	CC23, CC24, CC27, CC28, CC29, CC30, CC31, CC32, CC43, CC44	-	10	10
Wondogenet	-	CW25,CW26	-	2	2
Alemtena	-	CA02, CA45	-	2	2
Total			**51**	**30**	**81**

Table S2a. Carbon source utilization of cowpea and groundnut isolates and reference strains. (*Bradyrhizobium elkanii* (LMG 6164), *Bradyrhizobium japonicum* (HAMBI 2314^T), *Rhizobium giardinii* (HAMBI 2323^T), *Rhizobium tropici* (HAMBI 1163^T), *Ensifer meliloti* (HAMBI 2148^T), *Mesorhizobium mediterraneum (*HAMBI 2096^T), *Azorhizobium caulinodans (*HAMBI 216^T)).

Strain	Cluster	Carbon source																					
		Trehalose	Dulcitol	Raffinose	Maltose	Sorbitol	Arabinose	Citric acid	Malonic acid	Xylitol	Myo-inositol	Sucrose	Fructose	Starch	Inulin	Galactose	Dextrin	Xylose	Mannose	Ribose	Rhaminose	Mannitol	D-glucose
CA02	I	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CZ04	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH05	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH06	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH07	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH08	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH09	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH10	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CH11	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC23	VII	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC24	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CW25	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CW26	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC27	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC28	Un	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC29	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC30	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC31	IV	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC32	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CBo33	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CBo34	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CZ35	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CBo36		+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CBo37	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CBo38	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CZ41	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CZ42	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC43	III	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CC44	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CA45	IV	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GBa001	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ007	VI	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ012	VI	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ013	VI	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ014	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+

Table S2a. Contd.

Strain	Cluster	Carbon source																					
		Trehalose	Dulcitol	Raffinose	Maltose	Sorbitol	Arabinose	Citric acid	Malonic acid	Xylitol	Myo-inositol	Sucrose	Fructose	Starch	Inulin	Galactose	Dextrin	Xylose	Mannose	Ribose	Rhaminose	Mannitol	D-glucose
GZ015	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ017	VI	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ018	VI	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH019	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH020	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH021	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH022	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH023	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH024	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH025	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH026	VII	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ034	IV	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GZ035	IV	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG036	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG037	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG039	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG049	Un	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG055	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG058	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG060	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG064	III	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG065	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG068	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG072	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG074	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG077	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG078	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG081	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG083	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG086	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG087	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG088	I	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG089	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GG091	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH095	Un	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH096	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH097	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+

Table S2a. Contd.

Strain	Cluster	Carbon source																					
		Trehalose	Dulcitol	Raffinose	Maltose	Sorbitol	Arabinose	Citric acid	Malonic acid	Xylitol	Myo-inositol	Sucrose	Fructose	Starch	Inulin	Galactose	Dextrin	Xylose	Mannose	Ribose	Rhaminose	Mannitol	D-glucose
GH098	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH099	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH100	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH101	VII	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH102	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH103	I	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH104	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GH105	II	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
GBo126	V	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
HAMBI 216^{T}	Un	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
LMG 6164	II	+	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
HAMBI 2323^{T}	Un	+	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
HAMBI 1163^{T}	Un	+	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
HAMBI 2314^{T}	II	+	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
HAMBI 2148^{T}	Un	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
HAMBI 2096^{T}	Un	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+

Table S2b. Nitrogen source utilization, phosphate solubilizing ability (PSA) of the isolates and reference strains. (*Bradyrhizobium elkanii* (LMG 6164), *Bradyrhizobium japonicum* (HAMBI 2314^T), *Rhizobium giardinii* (HAMBI 2323^T), *Rhizobium tropici* (HAMBI 1163^T), *Ensifer meliloti* (HAMBI 2148^T), *Mesorhizobium mediterraneum (*HAMBI 2096^T), *Azorhizobium caulinodans (*HAMBI 216^T)).

		Nitrogen source																
Strain	**Cluster**	**L-aspargine**	**L-proline**	**L-leucine**	**L-alanine**	**Inosine**	**L-theronine**	**L-histidine**	**Uredine**	**L-phenylalanine**	**Thymidine**	**L-arginine**	**L-glutamic acid**	**L-pyroglutamic acid**	**Aspartic acid**	**D-serine**	**Glycine**	**PSA**
CA02	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CZ04	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CH05	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CH06	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CH07	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CH08	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CH09	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CH10	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CH11	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CC23	VII	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
CC24	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CW25	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CW26	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CC27	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CC28	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CC29	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CC30	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CC31	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
CC32	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CBo33	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CBo34	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CZ35	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CBo36	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CBo37	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CBo38	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
CZ41	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CZ42	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CC43	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
CC44	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CA45	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GBa001	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GZ007	VI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GZ012	VI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

Table S2b. Contd.

Strain	Cluster	Nitrogen source																
		L-aspargine	L-proline	L-leucine	L-alanine	Inosine	L-theronine	L-histidine	Uredine	L-phenylalanine	Thymidine	L-arginine	L-glutamic acid	L-pyroglutamic acid	Aspartic acid	D-serine	Glycine	PSA
GZ013	VI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GZ014	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GZ015	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GZ017	VI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GZ018	VI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GH019	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH020	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH021	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH022	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH023	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH024	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH025	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH026	VII	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GZ034	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GZ035	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG036	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GG037	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GG039	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG049	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG055	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG058	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG060	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG064	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
GG065	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG068	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG072	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG074	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG077	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG078	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG081	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG083	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG086	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GG087	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GG088	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
GG089	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

Table S2b. Contd.

Strain	Cluster	Nitrogen source																
		L-aspargine	L-proline	L-leucine	L-alanine	Inosine	L-theronine	L-histidine	Uredine	L-phenylalanine	Thymidine	L-arginine	L-glutamic acid	L-pyroglutamic acid	Aspartic acid	D-serine	Glycine	PSA
GG091	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GH095	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH096	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH097	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH098	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH099	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH100	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
GH101	VII	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
GH102	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH103	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH104	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GH105	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
GBo126	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HAMBI 216^{T}	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 6164	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HAMBI 2323^{T}	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HAMBI 1163^{T}	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HAMBI 2314^{T}	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
HAMBI 2148^{T}	Un	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
HAMBI 2096^{T}	Un	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-

academicJournals

Vol. 12(9), pp. 218-224, 7 March, 2018
DOI: 10.5897/AJMR2018.8794
Article Number: 0C8BBCD56217
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Virulence and antimicrobial susceptibility profile of *Listeria monocytogenes* isolated from frozen vegetables available in the Egyptian market

Rasha I. Mohamed [1], Mohamed A. Abdelmonem[1] and Heba M. Amin[2*]

[1]Department of Microbiology, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals, 7 Nadi El-said Street, Dokki, Giza, Egypt.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, MSA University, Cairo, Egypt.

Received 8 January, 2018; Accepted 6 February, 2018

Listeria monocytogenes **is among the most important foodborne pathogens. It may enter food-processing environments through raw materials, handlers or equipment and may persist due to ineffective cleaning or sanitation. The bacterium can be isolated from both frozen vegetables and fresh food substances. This study aimed to estimate the prevalence of *L. monocytogenes* in spices and frozen vegetables and screen for some virulence factors and drug-resistance determinants of the isolated bacteria. First, conventional microbiological methods were used for the isolation and identification of bacteria. Next, the identity of isolated bacteria was confirmed by molecular techniques, and the virulence genes *iap* and *hlyA* were identified by real-time polymerase chain reaction (PCR). The hemolytic activity of the isolates was assessed by cultivation on sheep blood agar. Furthermore, the antimicrobial susceptibility of confirmed *L. monocytogenes* isolates was tested by the disk diffusion method against 10 antibiotics. Out of 331 vegetable samples, 47 isolates were confirmed to contain *L. monocytogenes*, whereas none of 40 spice samples tested positive. All isolates were positive for *iap* and *hlyA* genes. Susceptibility testing indicated that all isolates were sensitive to trimethoprim/ sulfamethoxazole, but only 36% were sensitive to penicillin G, while 100% and 70% showed intermediate resistance to chloramphenicol and erythromycin, respectively. All tested isolates were resistant to amoxicillin, gentamicin and norfloxacin; on the other hand, 90, 86 and 84% of the tested strains were resistant to ciprofloxacin, ceftazidime/clavulanic acid and amikacin, respectively. In summary, *L. monocytogenes* isolates disseminated in frozen vegetable samples from the Egyptian market were highly virulent, entirely multiple-drug resistant and were enriched in iron-containing vegetables. Since *L. monocytogenes* is primarily pathogenic to humans and causes a life-threatening disease, there is a potential infection risk for people who usually deal with frozen vegetables before cooking. Hence, surveillance to *L. monocytogenes* in frozen products, together with implementation of tight measures would be valuable in preventing listeriosis, and are highly recommended.**

Key words: *Listeria monocytogenes,* virulence gene, antibiotic resistance.

INTRODUCTION

Bacteria of the genus *Listeria* are Gram-positive, facultative anaerobic and non-spore forming bacilli (Wong and Freitag, 2004). The genus is represented by eight major species: *Listeria monocytogenes*, *Listeria innocua*,

Listeria welshimeri, *Listeria grayi*, *Listeria seeligeri, Listeria ivanovii, Listeria marthii* and *Listeria rocourtiae*; recently (Weller et al., 2015) added new species are *Listeria booriae* and *Listeria newyorkensis.* The most medically relevant species, *L. monocytogenes,* is classified into 13 serotypes. Serotypes 1/2a, 1/2b, 1/2c and 4b strains are associated with human infections (Graves et al., 2010; Leclercq et al., 2010). Almost all major outbreaks of invasive listeriosis are due to serotype 4b strains (Salcedo et al., 2003). The ability of these bacteria to survive and grow over a wide range of environmental conditions, including high salt concentration, refrigeration temperature, and low pH, which makes them a potential hazard in foods (Ryser and Marth, 2007) and the ability of *L. monocytogenes* to persist in the environment is due to their capacity to form biofilms (Colagiorgi et al., 2016). This organism is a recognized foodborne pathogenic bacterium that causes many diseases, from mild gastroenteritis to severe blood and/or central nervous system infections, as well as abortion in pregnant women. Many studies have detected *L. monocytogenes* in fresh product samples and even in some minimally processed vegetables (Lopez, 2008; Zhu et al., 2017). However, *L. ivanovii* and *L. seeligeri* have been also rarely associated with disease in humans (Lopez, 2008). Listeriosis was responsible for 30% of foodborne deaths from 1996 to 2005 and had a high case fatality rate of 16.9% according to Food Net US (Barton et al., 2011). *L. monocytogenes* expresses a highly conserved pore-forming toxin known as listeriolysin O (LLO). LLO is a member of a large family of cholesterol-dependent cytolysins (CDCs) found in several bacterial pathogens (e.g., streptolysin O of *Streptococcus pyogenes* and alveolysin of *Bacillus alvei*). It is the primary virulence factor in *L. monocytogenes* and is essential for its pathogenesis (Tweten, 2005; Cossart et al., 1989). The entire infection cycle of *L. monocytogenes* is governed by multiple proteins, such as internalin A and internalin B (encoded by *inlA* and *inlB*), hemolysin (encoded by *hly*), phosphatidylinositol-specific phospholipase C (PI-PLC, encoded by *plcA*), phosphatidylcholine-specific phospholipase C (PC-PLC, encoded by *plcB)* and actin polymerization protein (encoded by *actA*) (Jaradat et al., 2002). *L. monocytogenes* is susceptible to many antibiotics; but multi-drug resistant isolates have been reported (Jaradat et al., 2002). Listeria species are generally susceptible to a wide range of antimicrobials, but the first multi resistant *L. monocytogenes* strain has been isolated in 1988. Since then, antibiotic-resistant *L. monocytogenes* isolates have been recovered from food, environment, and human listeriosis cases (Soni et al., 2014). Currently, a β-lactam antibiotic (e.g., ampicillin or penicillin) combined with an aminoglycoside (for example, gentamicin) is the reference therapy for human listeriosis, while the second choice of treatment is a combination of vancomycin, erythromycin and trimethoprim-sulfamethoxazole for pregnant women or patients allergic to β-lactams (Hof, 2004).

This study aimed to estimate the prevalence of *L. monocytogens* in spices and frozen vegetables, and screen for some virulence factors and drug-resistance determinants of the isolated bacteria.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Forty spices and 331 frozen food samples (45 okra, 16 carrot, 20 green beans, 57 artichoke, 36 Molokia, 8 spinach, 11 green peas, 61 strawberry, 11 grape leaves, 2 broad bean, 4 broccoli, 14 grape, 2 peach, 29 salad, 6 mixed vegetables, 7 pomegranates and 2 cauliflowers) were collected from the Egyptian market. *L. monocytogenes* was isolated according to the ISO 11290 method (ISO 11290, Technical committee ISO iTC 34, Food products).

Twenty-five grams of each food sample was weighed and mixed with 225 ml of half Fraser primary enrichment medium. The mix was incubated at 30 ±1°C for 24 ± 2 h. 0.1 ml of primary enrichment was transferred to a tube containing 10 ml Fraser broth. Then, this inoculated medium was incubated at 37°C for 4 ± 2 h. From the primary enrichment culture, a loopful (10 µl) was inoculated on the surface of Listeria Agar according to Ottaviani and Agosti medium (MERCK) (Ottaviani et al., 1997) and chromogenic listeria agar medium (OXOID) and were observed for typical *L. monocyogenes* colonies. The identity of the isolated colonies was further confirmed biochemically following the Microbact 12L scheme (Table 1).

Molecular identification of *L. monocytogenes* and detection of virulence genes

Real-time PCR was used to identify *Listeria* genus. DNA was extracted by Prep Man® Ultra according to manufacturer's protocol. Ten microliters of the supernatant was transferred to a new tube containing 90 µl of ultra-pure water, and then vortexd. The mixture was used as a DNA template for PCR. Real-time PCR mixture solution was prepared Using Promag™ custom kit (PROMAGA GMBH, Berlin, Germany) according to manufacturer procedure and then added into PIKO 96-well PCR (Thermo Fisher Scientific, Vantaa, Finland). Primers and probes used for the detection of *hlyA* and *iap* genes are listed in Table 2.

Hemolytic activity assay

Haemolysin was detected by culturing *L. monocytogenes* isolates on blood agar base supplemented with 5% defibrinated sheep blood. Blood agar plates were then incubated at 37°C for 24 h. Colonies producing clear zones of haemolysis were classified according to zone diameter of haemolysis as strong, intermediate and weak (ISO 11290-1-(2014).

*Corresponding author. E-mail: htmagdy@msa.eun.eg.

Table 1. Substrates and reactions in Microbact 12L system used to identify *Listeria monocytogenes*.

Well no.	Designation	Reaction principle	Negative	Positive
1	Esculin	Hydrolysis of Esculin	Yellow	Black
2	Mannitol	Utilization of specific sugars resulting in the production of acidic end products	Purple	Yellow
3	Xylose			
4	Arabitol			
5	Ribose			
6	Rhamnose			
7	Trehalose			
8	Tagatose			
9	Glucode-1-phosphate			
10	Methyl-D-glucose			
11	Methyl-D-mannose			
12	Haemolysis	Haemolysis of red blood cells	Red cell deposit	Clear zone

Table 2. Primers used in RTi-PCR assays for *L. monocytogenes* (Rodriguez et al., 2004).

Name	Target gene	Type	Sequence
hlyQF	*hlyA*	Forward primer	5'-CAT GGC ACC ACC AGC ATC T-3'
hlyQR		Reverse primer	5'-ATC CGC GTG TTT CTT TTC GA-3'
hlyQP		TaqMan_ Probe	5'-FAM-CGC CTG CAA GTC CTA AGA CGC CA-TAMRA-3'
iapQFa	*iap*	Forward primer	5'-AAT CTG TTA GCG CAA CTT GGT TAA-3'
iapQRa		Reverse primer	5'-CAC CTT TGA TGG ACG TAA TAA TAC TGT T-3'
iapQP		TaqMan probe	5'-FAM-CAA CAC CAG CGC CAC TAC GGA CG-TAMRA-3'

Antimicrobial susceptibility testing

Antibiotic susceptibility was determined by the Kirby-Bauer disc diffusion method (Bauer et al., 1966) as previously recommended by the National Committee for Clinical and Laboratory Standards (NCCLS, 2012). Four to five colonies were picked up from overnight cultures; then, a loopful was inoculated into sterile TSB (about 3-4 ml/tube), incubated for 2 to 4 h. The culture turbidity was adjusted to 0.5 McFarland (equal to 0.08 – 1 absorbance at wavelength 624 nm). Using a sterile cotton swab, the bacterial broth culture was streaked on Muller Hinton agar surface. The inoculum was left to dry for 3 to 5 min. Discs were placed individually on the agar surface with sterile forceps and then gently pressed down onto agar surface to provide uniform contact. Plates were allowed to diffuse for 2 h in a refrigerator then incubated at 37 ± 2°C for 18 to 24 h. The susceptibility of the *Listeria* isolates was detected by a clear zone around the discs. Results were interpreted according to the standardized interpretive chart by NCCLS (NCCLS, 2012). The antibiotics used were as follows: Penicillin G (PG 10), trimethoprim 1.25 µg + sulfamethaxazole 23.75 µg (TS25), erythromycin (E15), ciprofloxacin (CIP5), Amoxicillin (AML10), amikacin (AK30), norofloxacin (NOR 10 µg), gentamycin (GM 200 µg), ceftazidime + clavulanic acid (CAL40) and chloramphenicol (C30) (MAST Diagnostics-UK).

RESULTS

Distribution of *L. monocytogenes* in tested food samples

When forty spice samples and 331 frozen samples were examined for *L. monocytogenes,* 47 out of the 331 vegetable samples (14.2%) were positive for the presence of *L. monocytogenes* (Figure 1), while none of the spice samples were positive.

Haemolytic activity and frequency of virulence genes among *L. monocytogenes* isolates

All 47 *L. monocytogenes* isolates were PCR-positive for *iap* and *hlyA* genes. *L. monocytogenes* isolates showing haemolytic activity were classified according to their potency as shown in Figure 2.

Antimicrobial susceptibility of the *L. monocytogenes* isolates

The *in vitro* susceptibility of 47 *L. monocytogenes* strains isolated from different kinds of foods was tested against 10 antibiotics. All tested strains were sensitive to Trimethoprim/Sulfamethoxazole, while 36% of tested strains were sensitive to Penicillin G. Moreover, 100 and 70% of the samples showed intermediate resistance to Chloramphenicol and Erythromycin, respectively. All tested strains were resistant to Amoxicillin, Gentamicin and Norfloxacin, while 90, 86, 84% of tested strains were

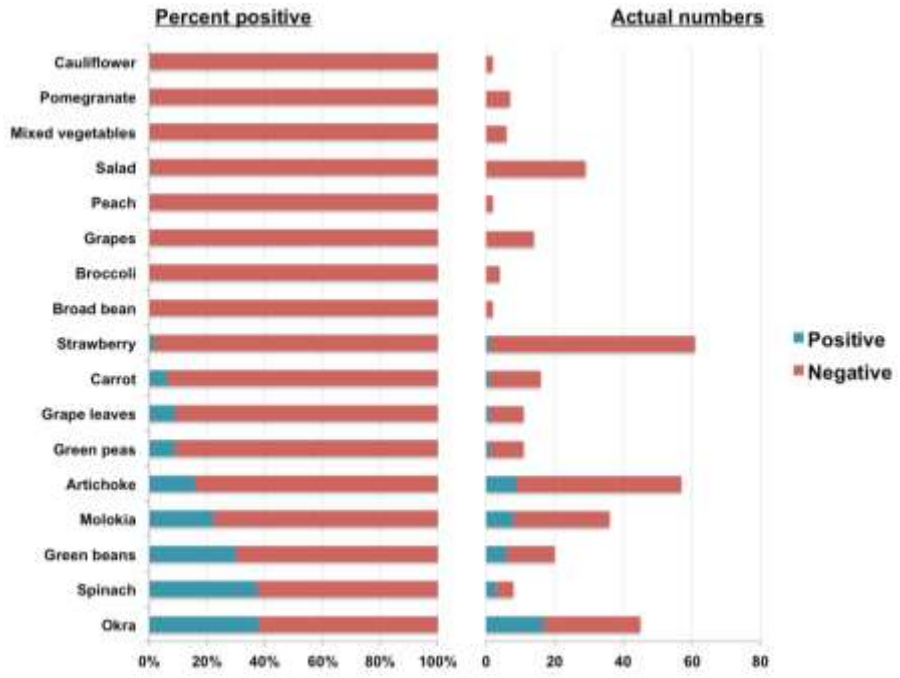


Figure 1. Distribution of isolated *L. monocytogenes* among vegetable samples.

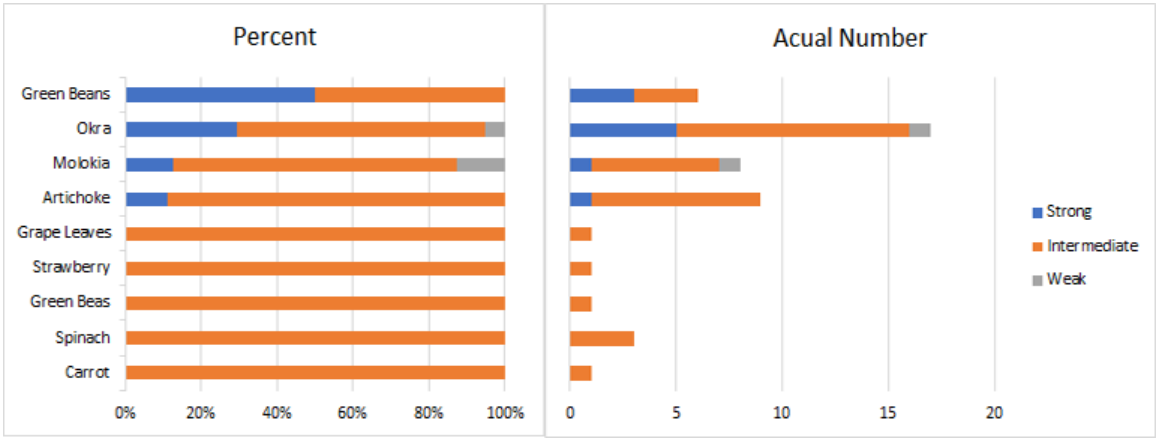


Figure 2. Hemolytic activity among *L. monocytogens* isolates.

resistant to Ciprofloxacin, Ceftazidime/clavulanic acid and Amikacin, respectively.

Statistical analysis

Chi-square tests were used to determine significant trends in the data. First, it was obvious from the culture results that *L. monocytogenes* cannot be isolated from spices (0% in 40 spices samples as opposed 14.2% of 331 frozen food samples).

Among the food samples, however, a clear over representation of *L. monocytogenes* was observed in okra, spinach, and artichoke with $p < 0.05$ which indicates statistically significant relationship between the categorical variables.

DISCUSSION

This study aimed to isolate *L. monocytogenes* from different kinds of spices and frozen vegetables. Overall, 40 spices and 331 vegetable samples were examined for the presence of *L. monocytogenes*. It was found that 47

(14.2%) samples out of 331 (17 okra,1 carrot, 6 green beans, 9 artichoke, 8 molokia, 3 spinach, 1 green peas, 1 strawberry and 1 grape leaves) were positive for *L. monocytogenes*. Meanwhile, surprisingly none of the spice samples showed any positive results for the pathogen. The absence of *Listeria* in spices may suggest a potential antimicrobial activity of these spices, and this will need confirmation in further studies. Even though reports on the sensitivity of *L. monocytogenes* to spices such as ginger, finger-root and turmeric were studied (Thongson et al., 2005), the current search for *L. monocytogenes* in spices was based on recent reports of detection of a number of food pathogens including *L. monocytogenes* in spices and herbs (Thongson et al., 2005; Kara et al., 2015).

Previous studies among the analyzed categories showed variation in occurrence of *L. monocytogenes*. For instance, Byrne et al. (2016) studied the occurrence and antimicrobial resistance patterns of listeria isolated from vegetables in Brazil and found that 3% of the samples were contaminated with *L. monocytogenes*, including 2% raw vegetables and 5.5% ready-to-eat vegetables. They confirmed the virulence potential of the isolates and antimicrobial susceptibility, revealing 50% of the isolates were susceptible to antibiotics (Byrne et al., 2016).

In Uruguay, on the other hand, 11.2% of different food samples were positive for *L. monocytogenes.* The highest percentage was among frozen food samples (38%) followed by cheese (10%). The same study discussed the serotype distribution among the samples and concluded on the prevalence of serotype 1/2b and 4b. These results highlight the role that frozen foods may play in the spread of this pathogen (Braga et al., 2017).

Moreover, the prevalence of *L. monocytogenes* in frozen burger patties was studied by Wong et al. (2012) in Malaysia. *L. monocytogenes* was detected in 33% of the chicken burger patties, 22.9% of the beef patties and 10% of fish patties; their results suggest that burger acts as a potential source of listeriosis if adequate cooking is not involved.

Finally, the prevalence of *Listeria* species in fresh and frozen fish and shrimp was studies in Iran by Rahimi et al. (2012). *Listeria* species were isolated in 7.5, 4.2, 11.7 and 6.6% of fresh fish, frozen fish, fresh shrimp and frozen shrimp, respectively. Almost 2% of identified species were *L. monocytogenes* which led to the conclusion that consumption of sea food either raw or frozen may lead to food borne illnesses in Iran (Rahimi et al., 2012).

L. monocytogenes detected in this study were positive for both *iap* and *hlyA* genes. Isolates showing haemolytic activity were classified according to their degree of heamolysis, into strong, intermediate and weak. Previous studies reported isolates positive for the virulence genes *inlA, inlB, prfA, iap, actA, plcB* and *hlyA*; their results suggest that all *L. monocytogenes* isolates have the potential to cause listeriosis in humans (Xiaolong et al., 2017). Various genes such as *hlyA* and *iap* genes have been targeted for detection of *L. monocytogenes* using PCR (Aznar et al., 2003). Pulsed field gel electrophoresis (PFGE) methodology is recommended in the identification protocol to identify the food implicated in an outbreak which is considered a key point for public health.

From previous reports, it is evident that differences in prevalence of *L. monocytogenes* in different types of food reflect the effect of geographical location, demography, and food type and hygiene standards among other factors. Food containing only spices or high levels of them, like Indian food, almost lack *L. monocyotogenes* (Suriyapriya et al., 2016). As indicated above, none of our 40 spice samples collected from the Egyptian market contained *Listeria*, agreeing with what was found in Indian spicy food (Suriyapriya et al., 2016).

Susceptibility testing results (Figure 3) indicates that all tested strains were multi drug resistant as they were resistant to amoxicillin, gentamicin and norfloxacin. Moreover, 90, 86 and 84% of the tested strains are resistant to ciprofloxacin, ceftazidime + clavulanic acid and amikacin, respectively.

In previous studies, all *L. monocytogenes* isolates were sensitive to most of the commonly used antibiotics, such as ampicillin, penicillin G and vancomycin. However, some multidrug-resistant *L. monocytogenes* isolates had been reported, which were resistant to ampicillin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole or rifampin. For example, a *L. monocytogenes* strain isolated from a meningoencephalitis patient was resistant to chloramphenicol, erythromycin, streptomycin and tetracycline (Charpentier et al., 1999).

These antibiotics have been increasingly used as supplements in animal feed, as growth promoters and for the treatment of human disease (Adzitey et al., 2013). Some common antibiotics, such as ampicillin, that are commonly used to treat clinical listeriosis, represent a high drug resistance phenomenon in *L. monocytogenes* strains. In recent years, with extensive use and abuse of antibiotics, multi-drug resistant strains have been detected from a variety of food samples (Ling et al., 2006). These findings confirmed that the prevalence of antibiotic resistance in *L. monocytogenes* might be increasing (Chen et al., 2014).

Conclusion

The findings of this study revealed a relatively high prevalence of virulent *L. monocytogenes* in frozen food in Egypt, which could potentially cause human disease. Thus, it is necessary to take precautions in the food factories, and periodical inspection must be performed on frozen food, which would be valuable to prevent human infection during consumption of this kind of food. All isolates recovered in this study were multi-drug resistant to most available antimicrobial agents, which represents

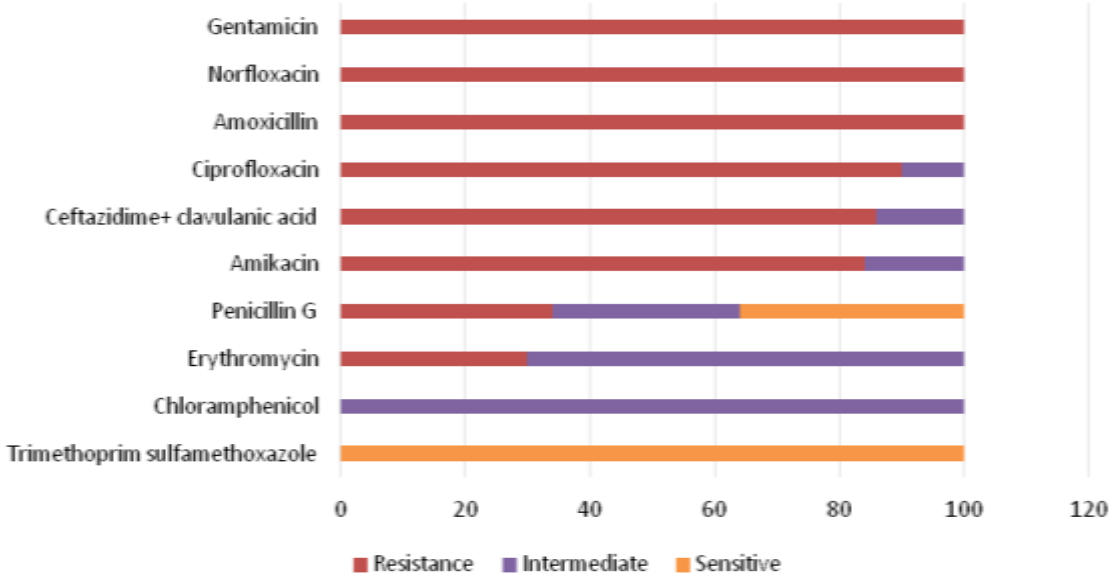


Figure 3. Percentage of sensitivity and resistance against 47 *L. monocytogenes* isolates.

a public health concern; thus, searching for alternatives is required.

This study is a full-scale, systematic investigation of the prevalence of *L. monocytogenes* in frozen foods in Egypt and the contamination of these foods, and it provides baseline information for Egyptian regulatory authorities to allow the formulation of a regulatory framework for controlling *L. monocytogenes* and to improve the microbiological safety of frozen foods.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Agriculture and Land Reclamation- Agriculture Research Center-Central Laboratory of Residue Analysis and Pesticides on Food in Egypt. The research was performed in the facilities of the microbiology laboratories of the Central Laboratory of Residue Analysis and Pesticides on Food.

REFERENCES

Adzitey F, Rahmat Ali GR, Huda N, Cogan T, Corry J (2013). Prevalence, antibiotic resistance and genetic diversity of Listeria monocytogenes isolated from ducks, their rearing and processing environments in Penang, Malaysia. Food Contr. 32:607-614.

Aznar R, AlarcónB(2003). PCR detection of Listeria monocytogenes: a study of multiple factors affecting sensitivity. J. Appl. Microbiol. 95:958-966.

Behravesh CB, Jones TF, Vugia DJ, Long C, Marcus R, Smith K, Thomas S, Zansky S, Fullerton KE, Henao OL, Scallan E (2011). Deaths associated with bacterial pathogens transmitted commonly through food: Foodborne Diseased Active Surveillance Network (FoodNet), 1996-2005. J. Infect. Dis. 204:263-267.

Bauer AW, Kirby WMM, Sherris JC, Turck M (1966). Antibiotic Susceptibility testing by a standardized single disc method. Amer J. Clin. Pathol. 45:493-496.

Braga V, Vázquez S, Vico V, Pastorino V, Mota M I, Legnani M, Varela G (2017). Prevalence and serotype distribution of Listeria monocytogenes isolated from foods in Montevideo-Uruguay. Brazilian. J. Microbiol. 48(4):689-694.

Byrne D V, Hofer E, Vallim DC, de Castro Almeida RC.(2016). Occurrence and Antimicrobial Resistance Patterns of Listeria Monocytogenes Isolated from Vegetables. Brazilian. J .Microbiol. 47(2):438-443.

Charpentier E, Courvalin P (1999). Antibiotic resistance in Listeria spp. Antimicrob. Agents Chemother; 43:2103-2108.

Chen M, Wu Q, Zhang J, Yan Z, Wang J (2014). Prevalence and characterization of *Listeria monocytogenes* isolated from retail-level ready-to-eat foods in South China. Food Control 2014: 38: 1-7

Colagiorgi A, Di Ciccio P, Zanardi E, Ghidini S, Ianieri A (2016). A Look inside the *Listeria monocytogenes* Biofilms Extracellular Matrix. *Microorganisms* 4(3):22.

Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Berche P(1989). Listeriolysin O is essential for virulence of Listeria monocytogenes:direct evidence obtained by gene complementation. Infect. Immun.57:3629-3636.

Dongyou L, Mark L, Jerald A, Frank WA (2005).Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and a virulent strains. FEMS Microbiol. Lett. 243(2):373-378.

Graves LM, Helsel LO, Steigerwalt AG, Morey RE, Daneshvar M I, Roof SE, Orsi RH, Fortes ED, Milillo SR, Den Bakker HC, Wiedmann M, Swaminathan B, Sauders BD (2010). Listeria marthii sp. nov., isolated from the natural environment, Finger Lakes National Forest. International J. Syst. Evol. Microbiol. 60:1280-1288.

Hof H (2004). an update on the medical management of listeriosis. Expert. Opin. Pharmacother. 5:1727-1735.

ISO 11290-1 (1996)/Amd 1. Modification of the isolation media and the haemolysis test, and inclusion of precession data. Geneva: International Organization for Standardization, International Electrotechnical Committee; 2004.

Jaradat ZW, Schutze GE, Bhunia AK (2002). Genetic homogeneity

among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. Int. J. Food Microbiol. 76:1-10.
Kara R, Gokmen M, Akkaya L, Gok V (2015). Microbiological Quality and Salmonella spp., /*Listeria monocytogenes* of Spices in Turkey. Res. J. Microbiol. 10(9):440-446.
Leclercq A, Clermont D, Bizet C, Grimont PA, Le Fle`che-Mate´os A, Roche SM, Buchrieser C, Cadet-Daniel V, Le Monnier A, Lecuit M, Allerberger F (2010). *Listeria rocourtiae* sp. Int. J. Syst. Evol.. Microbiol. 60:2210-2214.
Ling-ling M, Li-qiao L, Ming Z, Lin-hua W, Xue-xia P, Jun-yan Z (2006). Serum type and drug resistance of Listeria monocytogenes in food. Chin. J Health Lab. Technol. pp. 1165-1166.
Lopez J (2008). Listeria monocytogenes, in: OIE (Ed.), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.09.07_LISTERIA_MONO.pdf.
National Committee for Clinical and Laboratory Standards (NCCLS) (2012). Performance Standards for Antimicrobial Disc Susceptibility Tests: Thirty-Two Informational Supplement M100-S22.National Committee for Clinical Laboratory Standards Institute. Wayne, Pa, USA.
Ottaviani F, Ottaviani M, Agosti M (1997) Esperienza su un agar selettivo e differentiale perListeria monocytogenes. Industries Alimentarius 36:1-3.
Rahimi E, Shakerian A, Raissy M (2012). Prevalence of Listeria species in fresh and frozen fish and shrimp in Iran. Ann Microbiol. 62(1):37-40. doi:10.1007/s13213-011-0222-9.
Rodríguez-la D, Herna M, Scortti M, Esteve T (2004). by Real-Time PCR : Assessment of hly , iap , and lin02483 Targets and AmpliFluor Technol. 70(3):1366-1377. doi:10.1128/AEM.70.3.1366.
Salcedo C, Arreaza L, Alcala B, De La Fuente L, Vazquez JA (2003). Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. J. Clin. Microbiol. 41:757-762.
Soni DK, Singh M, Singh DV, Dubey SK (2014). Virulence and genotypic characteriza-tion of Listeria monocytogenes isolated from vegetable and soil samples. BMCMicrobiol. 14:1.
Suriyapriya S, Selvan P, Porteen K, Kannan SS (2016). Prevalence of Listeria spp. in Traditional Indian Dairy Products from Chennai Metropolis, Tamil Nadu. Procedia Food Sci. 6:230-234.
Thongson C, Davidson PM, Mahakarnchanakul W, Vibulsresth P (2005). Antimicrobial Effect of Thai Spices against Listeria Monocytogenes and Salmonella Typhimurium DT104. J. Food Protect. 68(10):2054-2058.
Tweten RK (2005). Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. Infect. Immun. 73:6199-6209.
Weller D, Andrus A, Wiedmann M, den Bakker HC (2015). *Listeria booriae* sp. nov. and Listeria newyorkensis sp. nov., from food processing environments in the USA. Int. J. Syst. Evol. Microbiol. 65(1):286-292.
Wong KY, Freitag NE (2004). *Listeria monocytogenes* invasion and intracellular growth. In: Lamont, R.J. (Ed.), Bacterial Invasion of Host Cells. Cambridge University Press, Cambridge.
Wong W C, Pui C F, Tunung R, Cheah Y K, Nakaguchi Y, Nishibuchi M, Son R (2012). Prevalence of Listeria monocytogenes in frozen burger patties in Malaysia. Int Food Res J. 19(4):1751-1756.
Xiaolong C, Yi W, Yan W, Changyun Y (2017). Isolation and characterization of Listeria monocytogenes from the black-headed gull feces in Kunming, China. J Infect Public Health. http://dx.doi.org/10.1016/j.jiph.2017.03.003
Zhu Q, Gooneratne R, Hussain M (2017) . Listeria monocytogenes in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. Foods 6(3):21. doi:10.3390/foods6030021

academicJournals

Vol. 12(9), pp. 225-229, 7 March, 2018
DOI: 10.5897/AJMR2018.8798
Article Number: 22F867256219
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Prevalence and virulence determinants of *Escherichia coli* isolated from raw cow's milk

Gamal Younis[1], Amal Awad[1*] and Reem Ghabour[2]

[1]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, 35516, Egypt.
[2]Gastro Enterology Surgery Center, Mansoura University, Mansoura, 35516, Egypt.

Received 9 January, 2018; Accepted 6 February, 2018

Raw milk plays an important role in the survival and transport of pathogenic bacteria including *Escherichia coli* strains. This study was performed to determine the existence of *E. coli* in raw milk intended for human consumption and its associated virulence determinants. In this context, a total of 232 milk samples were obtained from different dairy shops located at Mansoura city and its surrounding villages. Milk samples were subjected for bacteriological and serological examination of *E. coli*. Furthermore, *E. coli* strains were tested for its haemolytic activity on blood agar plates. The recovered *E. coli* strains were also screened by Polymerase chain reaction (PCR) for the presence of enterotoxins including heat –labile (LT), heat- stable (ST) toxins and haemolysin (*hly*) genes. The recovery rate of *E. coli* was 14.65% (34/232). Among the recovered *E. coli* strains, 12 different *E. coli* serotypes were identified namely, O26:H11 (n=6), O111:H2 (n=5), O128:H2 (n=5), O91:H21 (n=4), O124 (n=3), O127:H6 (n=3), O103:H21 (n=2), O153 (n=1), O113:H4 (n=2), O6 (n=1), O121:H7 (n=1) and O146 (n=1). Regarding PCR results, 31(91.19%) *E. coli* strains harbored *STa* and seven strains carried *hly* gene (20.59%) while non *E. coli* isoates harbord *LT* gene. Conclusively, raw milk can be considered as serious source of pathogenic *E. coli,* therefore, proper management practices and effective control measures are recommended to improve milk hygiene and sanitation.

Key words: Raw milk, *Escherichia coli*, enterotoxin genes, haemolysin gene.

INTRODUCTION

Raw milk harbor variable microorganisms, considered as an important source of food borne pathogens because it is regarded as perfect media for microbial growth (Laba and Udosek, 2013). Consumption of raw milk may be associated with the occurrence of food-poisoning outbreaks (Christidis et al., 2016).The presence of different food borne pathogens in milk may be contributed to the fecal contamination during milking process (Rehman et al., 2014). *E. coli* is a normal inhabitant of the gastrointestinal tract of both man and animals. Most of *E. coli* strains are harmless, but some are known to be pathogenic bacteria, causing severe intestinal and extra

*Corresponding author. E-mail: amalabdo@mans.edu.eg. Tel: +201003194875.

Table 1. Sequences and cycling conditions of oligonucleotide primers.

Primer	Sequence	Amplified product	Reference	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
hly	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177 bp	Piva et al. (2003)	94°C 5 min	94°C 30 s	60°C 1 min.	72°C 1 min	35	72°C 12 min
STa	GAAACAACATGACGGGAGGT GCACAGGCAGGATTACAACA	229 bp	Lee et al. (2008)	94°C 5 min	94°C 30 s.	57°C 30 s.	72°C 30 s	35	72°C 7 min
Lt	GGTTTCTGCGTTAGGTGGAA GGGACTTCGACCTGAAATGT	605 bp		94°C 5 min	94°C 30 sec	57°C 45 s	72°C 45 s	35	72°C 10 min

intestinal diseases in man (Croxen et al., 2013). Isolation of *E. coli* from milk represent a serious public health hazard because some strains of *E .coli* may be belongs to enteropathogenic or toxigenic or both groups which causes sever gastrointestinal disturbance (Thomas et al., 2017). Enterotoxigenic *E. coli* (ETEC) is one of the most common bacteria responsible for diarrhea in different parts of the world (Bagheri et al., 2014). Like other gastrointestinal infectious diseases, they are caused by lack of sanitation and most often contamination transfers from contaminated food or water (Walker et al., 2007; Marchou, 2013). There are two enterotoxins, Heat-stable toxin (ST) and Heat-labile toxin (LT). These two toxins are considered as the main virulence factors which influence the pathogenesis of ETEC strains (Kolenda et al., 2015; Sjöling et al., 2015). Alpha-hemolysin (HlyA) of *E. coli* is one of cytolytic pore-forming toxins (PFTs) produced by Gram-negative bacteria. *E. coli* HlyA lyses erythrocytes shows strong cytotoxic and cytolytic action against diversity of nucleated cells (Söderström et al., 2017). HlyA does not only kill and lyse cells but also affects target cells at sublytic concentrations. Haemolysin (*hlyA*) is produced mainly by extraintestinal pathogenic *E. coli* (ExPEC) strains and occasionally by ETEC, STEC and EPEC (Burgos and Beutin, 2010). Therefore, the main purpose of this study was to examine *E. coli* isolated from raw milk for the presence of enterotoxins including heat labile (LT) and heat stable (ST) toxins and haemolysin.

MATERIALS AND METHODS

Sampling

A total of 232 raw milk samples were collected randomly from different dairy shops, groceries and supermarkets in Mansoura city and its surrounding villages at Dakhalia Governorates, Egypt during the period from January to April, 2017. All samples were collected in sterile tubes and transported in an ice box to the laboratory as quick as possible for bacteriological examination with minimal of delay.

Isolation and identification of *E. coli*

All samples were immediately centrifuged and the sediment were streaked onto the surface of MacConkey's agar plates and incubated aerobically at 37°C for 24 h (Quinn et al., 2002). Lactose fermenting (Pink colored) colonies was sub-cultured on Eosin Methylene Blue (Oxoid) agar medium.

Colonies showing characteristic metallic green sheen on EMB agar were identified as *E. coli*. Presumptive *E. coli* colonies were subjected for gram staining and standard biochemical tests (Quinn et al., 2004). Additional identification of *E. coli* isolates was performed using commercial biochemical test kits (bioMerieux API, France).

Serological identification of *E. coli*

E. coli strains were transferred to Food Analysis Center, Faculty of Veterinary Medicine, Benha University for serological identification using rapid diagnostic *E. coli* antisera sets (Kok et al., 1996).

Haemolytic activity

E. coli isolates were cultured on blood agar containing 5% sheep blood, for detection of its haemolytic activity. Haemolysis was recorded after an overnight incubation at 37°C. A clear halo was defined as haemolysin positive (Brauner et al., 1990).

PCR assay for detection of enterotoxin genes (STa-LT) and haemolysin gene (*hly)*

Bacterial genomic DNA was extracted from *E. coli* isolates according to Ramadan et al. (2016). *E. coli* isolates were screened by Polymerase chain reaction (PCR) for the presence of enterotoxins (*Lt, STa*) and haemolysin (*hly*) genes. Oligonucleotide primers sequences and its amplicons sizes were described in Table1. Amplification reaction of PCR targeting haemolysin and enterotoxins was performed as previously described by Piva et al. (2003) and Lee et al. (2008), respectively (Table 1). Amplified DNA products for each gene were analyzed by 1.5% agarose gel electrophoresis in 1x TBE buffer stained with ethidium bromide visualized under UV transillumator.

RESULTS

In the present study, 34 (14.65%) *E. coli* strains have been recovered out of 232 examined milk samples. Among *E. coli* strains, twelve different *E. coli* serotypes were identified including, O26, O111, O128, O91, O124,

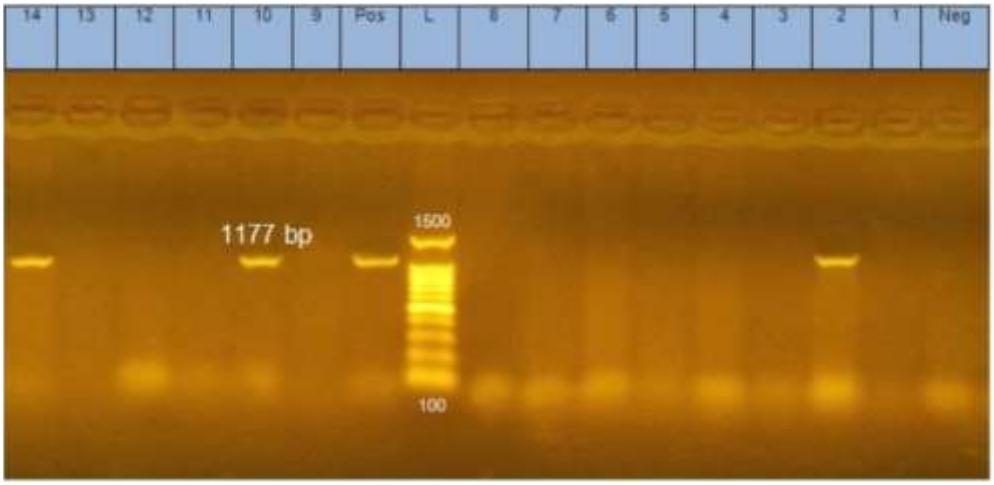


Figure 1. Agarose gel electrophoresis demonstrating amplification of *hly* gene at 1177 bp. Pos: Positive control, L: 100 bp DNA ladder, Neg: Negative control.

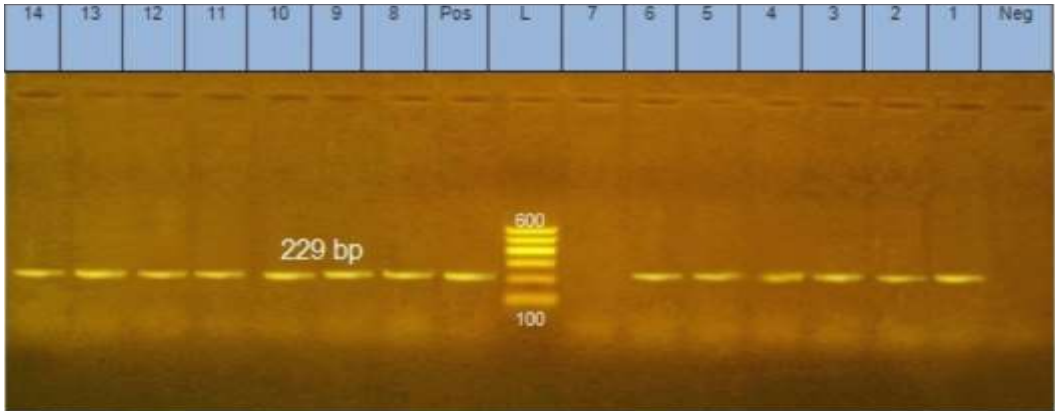


Figure 2. Agarose gel electrophoresis demonstrating amplification of STa gene at 229bp. Pos: Positive control, L: 100 bp DNA ladder, Neg: Negative control.

O127, O103, O113, O153, O6, O121 and O146 with a prevalence rate of 17.6, 14.7, 14.7, 11.7, 8.8, 8.8, 5.8, 5.8, 2.9, 2.9, 2.9 and 2.9% respectively.

E. coli isolates were tested for hemolytic activity on 5% sheep blood agar, 52.94% (18/ 34) of *E. coli* strains revealed different degrees of hemolysis. Based on the PCR results, 91.19% of *E. coli* isolates are potentially pathogenic, which carry one or more investigated virulence genes. From a total of 34 *E. coli* strains, 7(20.59%) strains carried *hly* gene (Figure 1), 31(91.17%) strains carried STa (Figure 2) while none *E. coli* isolates carried LT gene (Table 2).

DISCUSSION

Raw milk is a perfect medium that supports the growth and multiplication of *E. coli*. Consumption of such milk appeared as main threat to health concerns, especially for those people who still drink raw milk without heat treatment (Claeys et al., 2013). In the present study, *E.coli* was recovered with 14.65% prevalence rate. Similarly, *E. coli* has been isolated by several researchers from raw milk of cattle and buffaloes (Caine et al., 2014; Islam et al., 2008; Hossain et al., 2011). Compering to present results, a higher percentage of *E. coli* in milk was reported by Bandyopadhyay et al. (2012), Farzan et al. (2012), Mohd et al. (2013), Ali and Abdelgadir (2011) and Gwida and EL-Gohary (2013), who could isolate *E. coli* from raw milk in a percentage of 26.43,30.28, 33.96, 63 and 41.2% respectively. However, lower results were recorded by Kivaria et al. (2006) who detected *E. coli* in 6.3% of the examined raw milk samples.

In the present study, 12 different *E.coli* serotypes were identified; nearly the same serotypes were recovered

Table 2. Prevalence of serotypes, Enterotoxin (ST and LT) and haemolysin genes (hly) of *E. coli* isolated from raw milk.

Serotype (No)	Number (%)	hly gene (7)	Enterotoxin genes	
			LT	STa
O26:H11	6(17.6)	3	-	6
O111:H2	5(14.7)	-	-	5
O91:H21	4 (11.7)	-	-	4
O103:H21	2(5.8)	-	-	2
O113:H4	2(5.8)	-	-	-
O153	1(2.9)	1	-	1
O6	1(2.9)	1	-	1
O121:H7	1(2.9)	1	-	1
O146	1(2.9)	1	-	-
O124	3(8.8)	-	-	3
O127:H6	3(8.8)	-	-	3
O128:H2	5(14.7)	-	-	5
Total	34	7	-	31

from raw milk samples by Helmy et al. (2011) and Osman et al. (2012). Hemolysin is one of the important virulent factors in *E. coli*. In this study, 52.94% *E. coli* isolates revealed hemolysis on 5% sheep blood agar. Similarly, Farzan et al. (2012) reported that, one *E. coli* strain out of three isolates showed β-hemolytic activity on blood agar also, Lamey et al.(2013) found that 12.7% of isolated *E. coli* strains were hemolytic, Sayed (2014) found that one isolate (5.6%) out of 18 *E. coli* isolates had hemolytic activity. Concerning *hly* gene, 20.59% of *E. coli* harbored *hly* gene. A lower percentage was recorded by Ombarak et al.(2016), who identify *hly* gene in 2 (2.25%) isolates from karish cheese and one isolate (0.90%) from raw milk while, a higher percentages (42.85%) were reported by Osman et al. (2012). The presence of *E. coli* in milk especially enteropathogenic and/or toxigenic strains has a public health hazards which lead to sever gastrointestinal disturbance. Among *E. coli* isolates, 7(20.59%) strains carried *hly* gene, 31(91.17%) strains carried *STa* while, *LT* gene was not identified in all *E. coli* strains. Comparing to these results, Eid (2014) revealed that, only one strain were tested positive for *STa* gene out of eight E. *coli* isolates.

In Brazil, Paneto et al. (2007) studied the frequency of toxigenic *E. coli* in raw milk and cheese whereby, 1(2%) of *E. coli* isolates were ETEC. In Romania, Tabaran et al. (2017) analyzed 145 *E. coli* strains isolated from raw milk and traditional dairy cheeses, for the presence of *STa* and *STb*. In *LT,* none of the samples carries the *estI* gene, but 14 (9.7%) of the *E. coli* isolates were positive for both *eltA* and *estII*. Caine et al. (2014) examined 100 *E. coli* strain for the presence of enterotoxins which could identify enterotoxins in 4% of the total examined isolates.

Bonyadian et al. (2014) tested 120 isolates of *E. coli*, isolated from milk and unpasteurized cheeses which identified LT and STb in 2(1.66%) and 12(10.00%), respectively but could not identify *STa* gene. In this study, it was interesting that, all *E. coli* strains carry *hly* gene along with enterotoxin gene. These results suggest that food of animal origin represents a significant source of pathogenic *E. coli* strains.

Conclusion

The high contamination of milk with toxigenic *E. coli* represents a serious public health hazards which necessity high and strict preventive measures, to minimize the bacterial contamination within the food chain such as regular washing and sterilization of dairy equipment, utensils, milker's hands, animal udders as well as heat treatment of milk before distribution to consumers.

Significance statement

This study provided a data about the prevalence of *E. coli* in cow's raw milk, especially enterotoxigenic (ETEC) *E. coli*. These data is required for the establishment of food control systems which required the prevention and control of foodborne illnesses.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ali AA, Abdelghadir SW (2011).Incidence of *Escherichia coli* in raw cow's milk in Khartoum state.Br J. Dairy sci. 2(1):23-26.

Bagheri S, Mousavi Gargari SL, Rasooli I, Nazarian S, Alerasol M (2014). A CssA, CssB and LTB chimeric protein induces protection against Enterotoxigenic *Escherichia coli*. Braz. J. Infect. Dis. 18(3):308-314.

Bandyopadhyay S, Lodh C, Rahaman H, Bhattacharya D, Bera AK, Ahmed FA, Mahanti A, Samanta I, Mondal DK, Bandyopadhyay S, Sarkar S, Dutta TK, Maity S, Paul V, Ghosh MK, Sarkar M, Baruah KK (2012).Characterization of shiga toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC) in raw yak Poephagusgrunniens) milk and milk products. Res. Vet. Sci. 93(2):604-610.

Bonyadian M, Bonyadian M, Moshtaghi H, Taheri MA (2014). Molecular characterization and Antibiotic resistance of enterotoxigenic and entero-aggregative *Escherichia coli* isolated from raw milk and unpasteurized cheeses. Vet. Res. Forum. 5(1):29-34.

Brauner A, Katouli M, Tullus K, Jacobson SH (1990). Production of cytotoxic necrotizing factor, verocytotoxin and haemolysin by pyelonephritogenic *Escherichia coli*. Eur. J. Clin. Microbial Infect. Dis. 6:762-767.

BurgosY, Beutin L (2010). Common origin of plasmid encoded alpha-hemolysin genes in *Escherichia coli*. BMC Microbiol. 10:193.

Caine LA, Nwodo UU, Okoh AI, Ndip RN, Green E (2014). Occurrence of Virulence Genes Associated with Diarrheagenic *Escherichia coli* Isolated from Raw Cow's Milk from Two Commercial Dairy Farms in the Eastern Cape Province, South Africa. Int. J. Environ. Res. Public Health. 11(11):11950-11963.

Christidis T, Pintar KD, Butler AJ, Nesbitt A, Thomas MK, Marshall B, Pollari F (2016). Campylobacter spp. Prevalence and Levels in Raw Milk: A Systematic Review and Meta-Analysis. J. Food Prot. 79 (10):1775-1783.

Claeys WL, Cardoen S, Daube G, Block JD, Dewettinck K, Katelijne Dierick K, Zutter LD, Huyghebaert A, Imberechts H, Thiange P, Vandenplas Y, LieveHerman L (2013). Herman Raw or heated cow milk consumption: Review of risks and benefits. Food Contl. 31:251-262.

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin. Microbiol. Rev. 26:822-880.

Eid AM (2014). Molecular identification of some contagious microorganisms causing food poisoning from bulk tank milk in Gharbia Governorate. Benha Vet. Med. J. 27(2):29-47.

Farzan R, Rahimi E, Momtaz H (2012). Virulence properties of Shiga Toxin-Producing *Escherichia coli* isolated from Iranian raw milk and dairy products. Slov. Vet. Res. 49(4):159-66.

Gwida, MM, El-Gohary FA (2013). Zoonotic bacterial pathogens isolated from raw milk with special reference to *Escherichia coli* and *Staphylococcus aureus.* Governorate, Egypt. Open Access Sci. Rep. 2(4):705-708.

Helmy SM, Ammar MA, Aisha RA, El-Shabrawy MA , Hakim AS, Bakry MA, Abuelnaga, AS, Eraqi MM (2011). Molecular and virulence characterization of *Escherichia coli* strains isolated from persistent bovine mastitis. J. Am. Sci. 7:614-624.

Hossain Z, Sultana P, Deb S, Ahmed MM (2011). Multidrug resistance in large-plasmid-associated presumptive enterohaemorrhagic *Escherichia coli* isolated from contaminated lake water. Bangladesh J. Microbiol. 28:33-40.

Islam MA, Mondol AS, de Boer E, Beumer RR, Zwietering MH, Talukder KA, Heuvelink, AE (2008). Prevalence and genetic characterization of shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. Appl. Environ. Microbiol. 74:5414-5421.

Kivaria FM, Noordhuizen JPTM, Japaga AM (2006). Evaluation of the hygienic quality and associated public health hazards of raw milk marketed by small holder dairy producers in the Dar es Salaam region, Tanzania. Trop. Anim. Health Prod. 38(3):185-194.

Kok T, worswich D, Gowans E (1996). Some serological techniques for microbial and viral infections. In practical Medical Microbiology (collee, J.; Fraser ,A.; Marmion, B. and simmons A., eds.) 14thed ., Edinburg, Churchill Livingstone, UK.

Kolenda R, Burdukiewicz M, Schierack P (2015). A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. coli*. Front Cell Infect. Microbiol.5:23.

Laba SA, Udosek CE (2013). Bacteriological quality of raw cow milk in Ilorin, north central Nigeria. Nat. Sci. 11(10):73-79.

Lamey AE, Ammar AM, Zaki ER, Khairy N, Moshref BF, Refai MK (2013). Virulence factors of *Escherichia coli* isolated from recurrent cases of clinical and subclinical mastitis in buffaloes. Int. J. 4(1):86-94.

Lee SI, Kang SG, Kang ML Yoo HS (2008). Development of multiplex polymerase chain reaction assays for detecting enterotoxigenic *Escherichia coli* and their application to field isolates from piglets with diarrhea. J. Vet. Diagn. Invest. 20:492-496.

Marchou B (2013).Traveller's diarrhea: epidemiology, clinical practice guideline for the prevention and treatment. Presse Med. 42(1):76- 81.

Mohd R, Kotwal SK, Malik MA, Singh M (2013). Prevalence, genetic profile of virulence determinants and multidrug resistance of *Escherichia coli* isolates from foods of animal origin. Vet World. 6:139.

Ombarak RS, Hinenoya A, Awasthi Atsushi, SP, Iguchi A, Shima, A, Elbagory AM (2016). Yamasaki S. Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. Int. J. Food Microbiol. 221:69-76.

Osman KM, Mustafta KM, Aly AK AbdElhamed GS (2012). Serotypes, virulence genes, and intimin types of Shiga toxin-producing *Escherichia coli* and enteropathogenic *Escherichia coli* isolated from mastitic milk relevant to human health in Egypt. Vector Borne Zoonotic Dis. 12(4):297-305.

Paneto BR, Schocken-Iturrino RP, Macedo C, Santo E, Marin JM (2007). Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil. Arq. Bras. Med Vet. Zootec. 59:508-512.

Piva IC, Pereira AL, Ferraz LR, Silva RSN, Vieira AC, Blanco JE, Blanco M, Blanco J. Giugliano, LG (2003).Virulence Markers of Enteroaggregative *Escherichia coli* Isolated from Children and Adults with Diarrhea in Brasília, Brazil. J. Clin. Microbiol. 41(5):1827-1832.

Quinn PJ, Markey BK, Carter ME, Donnelly WJ Leonard FC (2002). Veterinary Microbiology and Microbial Diseases. 2ed. Blackwell Science Ltd. NdBodmin, Cornwall, UK.

Quinn J, Carter M E, Markey B Carter GR (2004). Clinical veterinary microbiology. 6th ed., Mosby, Edinburgh, London, New York, Philadelphia, St. Louis, Sydney, Toronto.

Ramadan H, Awad A, Ateya A (2016). Detection of phenotypes, virulence genes and phylotypes of avian pathogenic and human diarrheagenic *Escherichia coli* in Egypt. J. Infect. Dev. Ctries. 10(6):584-591.

Rehman MU, Rashid M, Sheikh JA, Bhat, MA (2014). Molecular epidemiology and antibiotic resistance pattern of enteropathogenic *Escherichia coli* isolated from bovines and their handlers in Jammu. India. J. Adv. Vet. Anim. Res. 1(4):177-181.

Sayed SM (2014). A contribution on Coliforms causing mastitis in cows with reference to serotypes and virulence factors of *E. coli* isolates. Ass. Univ. Bull. Environ. Res. 17(1):85-95.

Sjöling A, Mentzer, von A, Svennerholm AM (2015). Implications of enterotoxigenic *Escherichia coli* genomics for vaccine development. Expert. Rev. Vaccines 14 (4):551-560.

Söderström CM, Fagerberg SK, Brogaard MB, Leipziger J, Skals M, Praetorius HA (2017). Loop Diuretics Diminish Hemolysis Induced by α-Hemolysin from *Escherichia coli*. J. Membr .Biol. 250(3):301-313

Tabaran A, Mihaiu M, Tăbăran F, Colobatiu L, Reget O, Borzan M.M, Dan SD (2017). First study on characterization of virulence and antibiotic resistance genes in verotoxigenic and enterotoxigenic *E. coli* isolated from raw milk and unpasteurized traditional cheeses in Romania. Folia. Microbiol. (Praha). 62(2):145-150.

Thomas RR, Brooks HJ, O'Brien R (2017). Prevalence of Shiga toxin-producing and enteropathogenic *Escherichia coli* marker genes in diarrhoeic stools in a New Zealand catchment area. J. Clin. Pathol. 70(1):81-84.

Walker RI, Steele D, Aguado T (2007). Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *E. coli* (ETEC) disease. Vaccine 25(14):2545-2566.

academicJournals

Vol. 12(9), pp. 230-236, 7 March, 2018
DOI: 10.5897/AJMR2017.8798
Article Number: 2B8062956221
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Genotypic detection of the virulence factors of uropathogenic *Escherichia coli* isolated from diarrheic and urinary tract infected patients in Khartoum State, Sudan

Husam Eldin M. Hassan[1], Hisham N. Altayb[1], Mogahid M. El Hassan[2] and Miskelyemen A. Elmekki[2*]

[1]College of Medical Laboratory Science, Sudan University of Science and Technology, Sudan.
[2]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taibah University, KSA.

Received 24 November, 2017; Accepted 15 January, 2018

This study aimed to identify some important virulence factors, including *pap, fim, sfa, aer* and *hly* genes, typical of uropathogenic *Escherichia coli* (UPEC) in isolates collected from diarrheic and urinary tract infected patients in Khartoum State by multiplex polymerase chain reaction (PCR) assay. A total of 100 clinical specimens (50 urine and 50 diarrhea) were collected. Samples were cultured and identified by conventional method. Most study population were females 57/100 (57%); 42 suffering from urinary tract infections (UTIs) and 15 from diarrhea, while males were 43/100 (43%); 8 suffering from UTIs and 35 from diarrhea. Among enrolled subjects, 83 were positive for one or more uropathogenic *E. coli* virulent genes, while 17 isolates were negative for all genes. The results of multiplex PCR revealed that thirty two (n=32) diarrheal samples and fourteen (n=14) urine samples were *aer* positive. Thirty three (n=33) urine samples and eight (n=8) diarrheal samples appeared as *fim* positive. The genes *pap* and *hly* were found in 24 and 14 urine samples, respectively and in 9 and 3 diarrheal samples, respectively, while *sfa* gene was detected only in 15 urine specimens. The study concluded that *fim* gene was highly prevalent among UTI patients while *aer* gene was highly prevalent among diarrhea patients.

Key words: Uropathogenic *Escherichia coli*, *fimH*, aer, pap, sfa, hly, Sudan.

INTRODUCTION

Escherichia coli are a genetically diverse species that includes many pathotypes, both intestinal and extra-intestinal, most of which own specialized mechanisms characterized by their high efficiency in both colonization and pathogenicity. The appearance of different bacterial pathotypes is mainly due to horizontal transfer and exchange of genes responsible for virulence (Johnson, 2002).

E. coli possess genes encoding many pathogenicity associated factors including adhesions, siderophores

*Corresponding author. E-mail: mmekki@taibahu.edu.sa, miskatti@yahoo.com.

(that is, aerobactin), capsule and toxins implicated in urinary tract infection (UTI) pathogenesis. Several pathogenicity-associated genes were identified after the publication of the sequence of the whole genome of strain CFT073, a uropathogenic *E. coli* (UPEC). This strain was identified as the most cytotoxic that cause acute pyelonephritis as it was found to cause cytotoxicity in tissue culture cells in less than 18 h. Another strain that has been identified as invasive, the uropathogenic *E. coli* strain 536 (O6:K15:H31), which was isolated from acute pyelonephritis patient, this strain possess certain virulence factors; hemolysin (hly), P-related and S fimbriae (fim, prf, sfa), fimbrial adhesins type 1, the siderophores enterobactin (ent) and yersiniabactin (ybt) (Mobley et al., 1990). Moreover, acquisition of PIAs through horizontal gene transfer was also identified (Welch et al., 2002). Regardless of the common presence of type 1 fimbriae among Enterobacteriaceae, type 1 fimbriae may increase the virulence of UPEC for the urinary tract infection through several mechanisms including the promotion of bacterial persistence as well as enhancing the inflammation (Wullt, 2003).

Toxins produced by UPEC include hemolysin, cytotoxic necrotizing factor 1 (CNF1) and secreted auto transporter toxin (Sat) which has been shown to have a cytopathic effect on various bladder and kidney cell lines (Bahrani-Mougeot et al., 2002). In strain 536, four PAIs have been characterized which carry many pathogenicity associated genes (Dobrindt et al., 2002). The K15 capsule determinant of UPEC strain 536 is also found on a PAI (PAI V536) (Schneider et al., 2004). Some PAIs show genetic instability, whereas others appear to be relatively stable (Middendorf et al., 2004).

Some strains of *E. coli* considered as a common source of UTI and are able to colonise the vagina and found to originate from the lower GI tract (Czaja et al., 2009; Obata-Yasuoka et al., 2002). These strains usually originate from the stool, colonise the vagina and the periuretheral area through which they enter the UT (Salyers and Whitt, 2002). The presence of these bacteria in the UT without clinical symptoms is known as bacteriuria (Johnson, 1991; Mabbett, 2009). *E. coli* strains that colonise the UT may ascend towards bladder to cause cystitis and most probably, pyelonephritis, that may lead to kidney failure and death (Scholes et al., 2005).

The most accepted theory today is that UPEC germinated from non-pathogenic strains by gaining new virulence factors via accessory DNA horizontal transfer often organized into clusters (pathogenicity islands) located at chromosomal locus (Bahalo et al., 2013). The most common operons encoding P, S fimbriae are pyelonephritis associated pili (*Pap*) and S fimbrial adhesion (*sfa*) (Ribeiro et al., 2008). The pathogenicity of UPEC can be mediated by several virulence factors including bacterial adherence, in addition to the production of hemolysin and aerobactin (Arisoy et al., 2005). This study was performed to determine the virulence factors of UPEC in Khartoum.

MATERIALS AND METHODS

One hundred *E. coli* isolates were obtained from 100 clinical samples (50 urine, 50 diarrhea) collected from patients attending different hospitals in Khartoum state complaining from urinary tract infection and diarrhea, in the period from March to May, 2017. Urine samples were cultured on CLED agar, while diarrhea samples were cultured on MacConkey agar. Biochemical tests including indole as a key test, then urease, citrate and Kliger Iron Agar (KIA) test (according to CLSI guidelines) were used for identification of bacteria, in addition to the lactose fermentation on the MacConkey agar (Bahalo et al., 2013).

Bacterial isolates were grown on nutrient agar for an overnight then used for DNA extraction by boiling method (Yamamoto et al., 1995).

Polymerase chain reaction

Specific primers from Macrogen (Korea) were used to amplify the *fimH, pap, sfa, hly* and *aer* genes as shown in Table 1. The multiplex PCR assay was carried out in a total volume of 25 µL of mixture containing 2 µL Maxime PCR Premix (iNtRON, Korea) containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 1 U Taq DNA polymerase, 0.5 µL of each of the virulence gene-specific primers, forward and reverse primers for each gene (a total of 5 µL for the 5 target genes), 2 µL of template DNA and 16 µL of deionized water. The amplification conditions included three steps: heating at 94°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and the final extension at 72°C for 7 min (Jalali et al., 2015). Amplification was done using TECHNE® Ltd. peltier thermal cycler (Germany).

Visualization of the PCR products

The PCR product was visualized on 1.5% agarose gel in TBE buffer, 100-bp DNA ladder (iNtRON, Korea) was used to determine product size.

Data analysis

Statistical Package for Social Science Program (SPSS) version (11.5) was used for data analysis, *p*-value of less than 0.05 was considered significant (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp, Released, 2012).

RESULTS

A total of 100 patients (50 patients suffering from UTIs and 50 patients suffering from diarrhea) attending Khartoum hospitals during March to May, 2017, were enrolled in this study. Table 2 shows the association between UPEC virulence genes and gender while the association between UPEC virulence genes and the type of clinical specimen is shown in Table 3. The results of agarose gel electrophoresis for multiplex PCR are as shown in Figure 1A and B.

Table 1. Primers used for detection of virulence genes of UPEC strains.

Identified gene	Primer	Primers sequence (5-3)	Product size (bp)
papE/F	*pap3* *pap4*	GCAACAGCAACGCTGGTTGCATCAT AGAGAGAGCCACTCTTATACGGACA	336
fimH	*fim1* *fim2*	GAGAAGAGGTTTGATTTAACTTATTG AGAGCCGCTGTAGAACTGAGG	508
sfaD/E	*sfa1* *sfa2*	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410
Aer	*aer1* *aer2*	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602
hlyA	*hly1* *hly2*	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177

Jalali et al., 2015.

Table 2. Association between the presence of UPEC virulence genes and gender.

Gene	*pap*		*fimH*		*sfa*		*aer*		*hly*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Male	11	32	11	32	3	40	21	22	6	37	43
Female	22	35	30	27	12	45	25	32	11	46	57
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.171		0.006		0.051		0.621		0.481		-

Table 3. Frequency of UPEC virulence genes in urine and diarrhea samples.

Gene	*pap*		*fimH*		*sfa*		*aer*		*hly*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Urine	24	26	33	17	15	35	14	36	14	36	50
Diarrhoea	9	41	8	42	0	50	32	18	3	47	50
Total	33	67	41	59	15	85	46	54	17	83	100
P-value	0.001		0.000		0.000		0.000		0.003		-

There was significant association between the presences of *pap* gene and gentamicin, ciprofloxacin and co-trimoxazole susceptibility testing (p-value = 0.000, 0.039 and 0.035, respectively) and relatively significant to Amikacin (p-value= 0.068). Also there was significant association between the presences of *hly* gene and amikacin, ciprofloxacin and co-trimoxazole susceptibility testing (p-value = 0.002, 0.002 and 0.041, respectively) and relatively significant to gentamicin (p-value = 0.073) *fimH* gene statistically associated with co-trimoxazole susceptibility testing (p-value = 0.042) (Table 4). The frequency of virulence genes in stool and urine specimens is shown in Table 5, the most frequent multiple genes present in one isolate were pap an *fimH* genes in both urine and stool samples. However, when a single virulence gene frequency was considered, it was found that *fimH* is the most frequent among urine isolates while *aer* is the most frequent among stool isolates.

DISCUSSION

The gene of importance as indicated by the results of the present study, was shown to be *fimH,* which is present

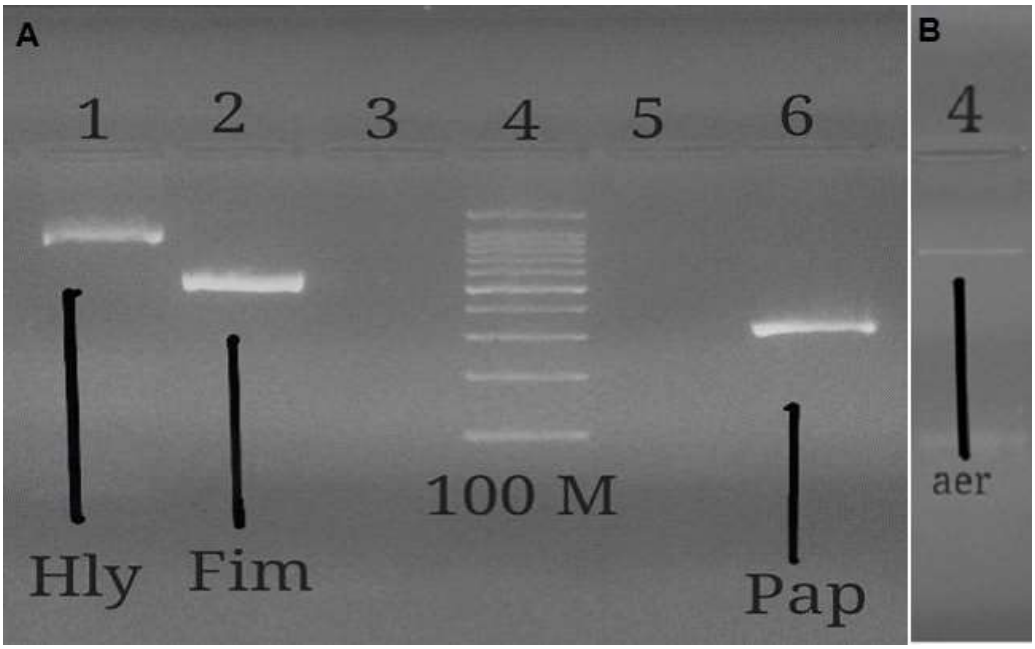


Figure 1. Agarose gel electrophoresis of multiplex PCR product: A; 1= positive *hly* gene, 2: positive *fimH* gene, 3, 5: negative samples, 4: 100 bp ladder, 6: positive *pap* gene, B: positive *aer* gene

Table 4. The association between the presence of UPEC virulence genes and antimicrobial susceptibility testing.

Gene		*Pap*		*fim*		*Sfa*		*aer*		*hly*		Total
Antibiotics		**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	
gentamicin	S	10	45	21	34	9	46	23	32	6	49	55
	R	23	22	20	25	6	39	23	22	11	34	45
	P-value	0.000		0.526		0.673		0.354		0.073		
Amikacin	S	30	66	38	58	14	82	43	53	14	82	96
	R	3	1	3	1	1	3	3	1	3	1	4
	P-value	0.068		0.158		0.568		0.235		0.002		
ciprofloxacin	S	14	43	21	36	5	52	23	34	4	53	57
	R	19	24	20	23	10	33	23	20	13	30	43
	P-value	0.039		0.330		0.045		0.192		0.002		
Co-trimoxazole	S	16	47	21	42	7	56	29	34	7	56	63
	R	17	20	20	17	8	29	17	20	10	27	37
	P-value	0.035		0.042		0.155		0.993		0.041		

+ve: Positive, -ve: negative, S: sensitive; R: resistant.

with high frequency (66%) in urine isolates as compared to the rest of the genes; this may indicate its essential role in *E. coli* causing UTI among Sudanese patients. It is well documented that type 1 pili are important for the invasion and persistence of the UPEC in the urinary bladder after its colonization. Type 1 pili colonization is enhanced by adhesin FimH (Hannan et al., 2012) encoded by fimH gene which recognizes certain α1β3 integrins (Guiton et al., 2012; Eto et al., 2007). It was also confirmed that FimH is important in pathogenesis and that the pathogenicity of a UPEC strain depends greatly on the ability of FimH to switch between conformations and this is also dependent on the different alleles that can be expressed by this gene, affecting FimH conformation

Table 5. Frequency of virulence gene(s) of *E. coli* isolated from urine and stools specimens.

Source of isolate	Number of genes	Number of isolate	*pap*	*fimH*	*sfa*	*aer*	*Hly*
Urine N=44	Four genes N=7	2	+	+	+	+	
		4	+	+	+	-	+
		1	+	-	+	+	+
	Three genes N=11	4	+	+	+	-	-
		1	+	-	+	+	-
		5	+	+	-	-	+
		1	-	+	-	+	+
		4	+	+	-	-	-
	Two genes N=13	1	-	+	+	-	-
		3	-	+	-	+	-
		2	+	-	-	+	-
		1	+	-	-	-	+
		2	-	-	-	+	+
	One gene N=13	9	-	+	-	-	-
		2	-	-	+	-	-
		2	-	-	-	+	-
	Three genes N=1	1	+	+	-	+	-
Stool N=39	Two genes N=11	3	+	+	-	-	-
		4	+	-	-	+	-
		1	+	-	-	-	+
		1	-	+	-	+	-
		2	-	-	-	+	+
	One gene N=27	24	-	-	-	+	-
		3	-	+	-	-	-

+: Positive; -: Negative.

and function (Schwartz et al., 2013). These results agree with published reports, which emphasize the predominance of fimbriae type 1 among the UPEC strains (Jalali et. al., 2015; Tarchouna et al., 2013; Usein et al., 2001). One important virulence factor of *E. coli* causing UTI is fimbriae-mediated adherence. While the role of type 1 fimbriae in virulence is unknown, it is well defined that specific adherence and increased induction of mucosal inflammation are the mechanisms used by P fimbriae to increase the virulence of UPEC (Connell et al., 1996). Significant association (p-value 0.006) was found between *fimH* gene and gender; this association may be due to difference in anatomical structure of urinary tract between male and female (Hickling et al., 2015).

The results confirmed the existence of *aer* gene with more prevalence in diarrheal isolates, this finding totally agreed with Oswald et al. (1991), who found that *aer* gene was positive in 70% of diarrheal samples and Micenková et al. (2014) who found *aer* gene was positive in 68%. High frequency of *aer* gene in diarrheal isolates may be attributed to the deficiency of iron concentration within gastrointestinal tract, while iron is responsible for microbial metabolism. Excretion of siderophores, such as enterobactin is a method by which most *E. coli* can increase access to iron. Aerobactin, salmochelin, and yersiniabactin are three other siderophores linked with pathogenesis, produced by a smaller proportion of isolates (Meyrier, 1999). Prevalence of aerobactin, which confers the ability to bind iron among isolates, was similar to those reported by other investigators in diarrhea isolates. The result show 64% *aer* gene positive in 50 diarrhea isolates and this percentage is near to other researches (Oswald et al., 1991) result of 70% *aer* gene positive and Micenková et al. (2014) result is 68% *aer* gene positive.

The result shows 24/50 (48%) *pap* gene positive in 50 urine isolate and this result is not far from Jalali et al. (2015) who found 46% *pap* positive gene, and Tarchouna et al. (2013), who found 41% *pap* positive gene. Pyelonephritis associated pili (*pap*) play an important role in the pathophysiology of pyelonephritis caused by *E. coli* (Tarchouna et al., 2013). UPEC colonize the bladder by binding urinary tract endothelial cells through utilization of P fimbria that bind D-galactose-D-galactosemoieties on

uroepithelial cells (Todar, 2007).

The result shows 14/50 (28%) *hly* gene positive in 50 urine isolate and this percentage is less than the result of Jalali et al. (2015) who found (47%) *hly* positive gene. There was a clear relation between tissue damage and the presence of hemolysin. Prevalence of these genes differs on the basis of phylogenetics, geographical distribution, and clinical presentation (Oliveira et al., 2011; Blanco et al., 1997). A huge variation in the frequencies of these genes was recorded worldwide (Abe et al., 2008).

When antibiotic resistance was investigated among the virulent isolates, a significant relation was observed since strains carrying the virulence genes were more resistant to several antibiotics. This agrees with a previous study from Iran (Derakhshandeh et al., 2015).

Eighteen isolate (18%) of *E. coli* were negative for UPEC virulence genes and sensitive to all antibiotics used in this study, 12 of them were in diarrheal samples and 6 were in urine samples. These negative isolates may be part of the normal flora that lack these virulent genes or may be due to the possibility of corresponding gene mutations, as negative PCR does not indicate the absence of the corresponding operon while a positive PCR usually confirms the presence of the virulence genes (Tarchouna et al., 2013).

Conclusion

The present study results showed that the UPEC strains isolated in Sudan have a different virulence profile when compared with other studies and it seems that the virulence of UPEC strains depends on the regional geography and climate. However, a recent study in Egypt showed similar distribution of virulence genes (Morsi and Elsaid Tash, 2016). This indicates that some social and environmental factors may contribute in the virulence pattern of UPEC in different communities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abe CM, Salvador FA, Falsetti IN, Vieira MA, Blanco J, Blanco JE, Blanco M, Machado AM, Elias WP, Hernandes RT, Gomes TA (2008). Uropathogenic *Escherichia coli* (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli.* FEMS Immunol. Med. Microbiol. 52:397-406.

Arisoy M, Aysev D, Ekim M, Özel D, Köse SK, Özsoy ED, Akar N (2005). Detection of virulence factors of *Escherichia coli* from children by multiplex PCR. Int. J. Clin. Prac. 60 (2):170-173.

Bahalo S, Tajbakhsh E, Tajbakhsh S, Momeni M, Tajbakhsh F (2013). Detection of some virulence factors of *Escherichia coli* isolated from urinary tract infection isolated of children in Shahrekord Iran by Multiplex PCR. Middle-East J. Sci. Res. 14(1):29-32.

Bahrani-Mougeot F, Gunther NW, Donnenberg MS, Mobley HLT (2002). "Uropathogenic *Escherichia coli*." In *Escherichia coli:* Virulence Mechanisms of a Versatile Pathogen. MS Donnenberg (ed). London: Academic Press. pp. 239-268.

Blanco M, Blanco JE, Rodrıguez E, Abalia I, Alonso MP, Blanco J (1997). Detection of virulence genes in uropathogenic *Escherichia coli* by PCR comparison with results obtained using phenotypic methods. J. Clin. Micro. 31:37-43.

Connell I, Agace W, Klemm P, Schembri M, Mărild S and Svanborg C (1996). Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. Proc. Nat. Acad. Sci. 93(18):9827-9832.

Czaja CA, Stamm WE, Stapleton AE, Roberts PL, Hawn TR, Scholes D, Samadpour M, Hultgren SJ, Hooton TM (2009). Prospective cohort study of microbial and inflammatory events immediately preceding *Escherichia coli* recurrent urinary tract infection in women. J. Infect. Dis. 200:528-536.

Derakhshandeh A1, Firouzi R, Motamedifar M, Arabshahi S, Novinrooz A, Boroojeni AM, Bahadori M, Heidari S (2015). Virulence Characteristics and Antibiotic Resistance Patterns among Various Phylogenetic Groups of Uropathogenic *Escherichia coli* Isolates. Jpn. J. Infect. Dis. 68(5):428-431.

Dobrindt U, Blum-Oehler G, Nagy G, Schneider G, Johann A, Gottschalk G, Hacker J (2002). "Genetic structure and distribution of four pathogenicity islands (PAI I536 to PAI IV536) of uropathogenic *Escherichia coli* strain 536." Infect. Immunity 70:6365-6372.

Eto DS, Jones TA, Sundsbak JL, Mulvey MA (2007). Integrin-mediated host cell invasion by type 1-piliated uropathogenic *Escherichia coli*. PLoS Pathog; 3:e100

Guiton PS, Cusumano CK, Kline KA, Dodson KW, Han Z, Janetka JW, Henderson JP, Caparon MG, Hultgren SJ (2012). Combinatorial small-molecule therapy prevents uropathogenic *Escherichia coli* catheter-associated urinary tract infections in mice. Antimicrob Agents Chemother. 56:4738-4745.

Hannan TJ, Totsika M, Mansfield KJ, Moore KH, Schembri MA, Hultgren SJ (2012). Host–pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection. FEMS Microbiol. Rev. 36:616-648.

Hickling DR, Sun TT, Wu XR (2015). Anatomy and Physiology of the Urinary Tract: Relation to Host Defense and Microbial Infection. Microbiology spectrum 3(4): doi: 10.1128/microbiolspec.UTI-0016-2012.

IBM SPSS Statistics for Windows, Version 21.0. (2012). Armonk, NY: IBM Corp, Released. www.ibm.com/software/analytics/spss/ IBM Corp.

Jalali HR, Pourbakhsh A, Fallah F, Eslami G (2015). Genotyping of Virulence Factors of Uropathogenic Escherichia coli by PCR. Novelty in Biomed. 3(4):177-181.

Johnson JR (2002) "Evolution of pathogenic *Escherichia coli*." In *Escherichia coli:* Virulence Mechanisms of a Versatile Pathogen. M. S. Donnenberg(ed). London: Academic Press, pp. 55-77.

Johnson JR (1991). Virulence factors in *Escherichia coli* urinary tract infection. Clin Microbiol Rev. 4:80-128.

Mabbett AN, Ulett GC, Watts RE, Tree JJ, Totsika M, Cheryl-lynn YO, Wood JM, Monaghan W, Looke DF, Nimmo GR, Svanborg C (2009). Virulence properties of asymptomatic bacteriuria *Escherichia coli* . Int. J. Med. Microbiol. 299:53-63.

Meyrier A (1999). Urinary Tract Infection. In: Schrier RW, Cohen AH, Glassock RJ, Grünfeld JP. Atlas of diseases of the kidney (PDF). 2. *Oxford: Blackwell Sci*. ISBN 0-632-04387-3.

Micenková L, Štaudová B, Bosák J, Mikalová L, Littnerová S, Vrba M, Ševčíková A, Woznicová V, Šmajs D (2014). Bacteriocin-encoding genes and ExPEC virulence determinants are associated in human fecal *Escherichia coli* strains. BMC Microbial. 14(1):109.

Middendorf B, Hochhut B, Leipold K, Dobrindt U, Blum-Oehler G, Hacker J (2004) "Instability of pathogenicity islands in uropathogenic *Escherichia coli* 536." J. Bacteriol.186:3086-3096.

Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell, CV, Jones BD dan Warren JW (1990). Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infect. Immunol. 58:1281.

Morsi SS, Tash RM (2016). Virulence Determinants among Extended-Spectrum B- Lactamases producers of Uropathogenic *Escherichia coli* isolates In Zagagig University Hospitals, Egypt, Egyptian J. Med. Microbiol. 25(2):101-108.

Obata-Yasuoka M, Ba-Thein W, Tsukamoto T, Yoshikawa H, Hayashi H (2002). Vaginal *Escherichia coli* share common virulence factor profiles, serotypes and phylogeny with other extraintestinal *E. coli*. Microbiology 148:2745-2752.

Oliveira FA, Paludo KS, Arend LN, Farah SM, Pedrosa FO, Souza EM, Surek M, Picheth G, Fadel-Picheth CM (2011). Virulence characteristics and antimicrobial susceptibility of uropathogenic *Escherichia coli* strains. Genet. Mol. Res. 10(4):4114-25.

Oswald E, de Rycke J, Lintermans P, van Muylem K, Mainil J, Daube G, Pohl P (1991). Virulence factors associated with cytotoxic necrotizing factor type two in bovine diarrheic and septicemic strains of *Escherichia coli*. J. Clin Microbiol. 29:2522-2527.

Ribeiro TIBA M, Yano T, Da Silva LEITE D (2008). Genotypic characterization of virulence factors in *Escherichia coli* strains from patients with cystitis. Rev Inst. Med. Trop. S Paulo. 50(5):255-60.

Salyers AA, Whitt DD (2002). Bacterial pathogenesis: a molecular approach. Washington DC: ASM Press.

Schneider G, Dobrindt U, Brüggemann H, Nagy G, Janke B, Blum-Oehler G, Buchrieser C, Gottschalk G, Emödy L, Hacker J (2004) "The pathogenicity island-associated K15 capsule determinant exhibits a novel genetic structure and correlates with virulence in uropathogenic *Escherichia coli* strain 536. Infect. Immun. 72:5993-6001.

Scholes D, Hooton TM, Roberts PL, Gupta K, Stapleton AE, Stamm WE (2005). Risk factors associated with acute pyelonephritis in healthy women. Ann. Int. Med.142:20-27.

Schwartz DJ, Kalas V, Pinkner JS, Chen SL, Spaulding CN, Dodson KW, Hultgren SJ (2013). Positively selected fimH residues enhance virulence during urinary tract infection by altering fimH conformation. Proc Natl. Acad. Sci. USA.; 110:15530-15537.

Tarchouna M, Ferjani A, Selma WB, Boukadida J (2013). Distribution of uropathogenic virulence genes in *Escherichia coli* isolated from patients with urinary tract infection. Int. J. Infect. Dis.17:450-3.

Todar K (2007). Pathogenic *E. coli*, in *Online Textbook of Bacteriology*, University of Wisconsin-Department of Bacteriology.[homepage on internet] cited on -11-30. Available from http://www.textbookofbacreiology.net/.

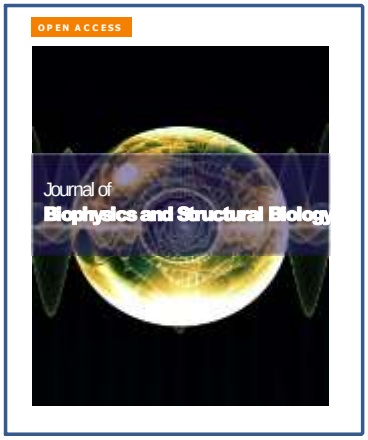
Usein CR, Damian M, Tatu-Chitoiu D, Capusa C, Fagaras R, Tudorache D, Nica M, Bouguénec C (2001). Prevalence of virulence genes in *Escherichia coli* strains isolated from Romanian adult urinary tract infection cases. J. Cell. Mol. Med. 5(3):303-310.

Welch RA, Burland V, Plunkett GI, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D (. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proceedings of the National Academy of Sciences. 99(26):17020-17024.

Wullt B (2003). "The role of P fimbriae for *Escherichia coli* establishment and mucosal inflammation in the human urinary tract." Int. J. Antimicrob. Agents 21:605-621.

Yamamoto S, Terai A, Yuri K, Kurazono H, Takeda Y, Yoshida O (1995). Detection of urovirulence factors in *Escherichia coli* by multiplex PCR. FEMS Immuno. Med. Microbiol. 12:85-90.

Related Journals:



academicJournals

www.academicjournals.org