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Bacterial Flora of the *Anopheles fluviatilis* S. L., the Vector of Malaria in Southern Iran for Proper Candidate Paratransgenesis

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A B S T R A C T

Malaria is considered most important health problems in the world. The disease was distributed in the south and southeast of Iran. At the present, Iran is in malaria elimination phase and new control tool need to vector control program. This study was carried out to find the bacteria for paraterasgenesis strategy as a new control method to cut the parasit life cycle. The microflora of the outer surface and gut of various stages of *Anopheles fluviatilis* James as one of the important malaria vector was studied using biochemical and molecular techniques during 2013-2014. Twelve bacteria species were found including; *Providencia rettgeri*, *Morganella morganii*, *Enterobacter aerogenes*, *Pseudomonas oryzihabitans*, *Citrobacter braakii*, *Citrobacter freundii*, *Aeromonas hydrophila*, *Klebsiella oxytoca*, *Citrobacter koseri*, *Serratia fonticola*, *Enterobacter sakazakii* and *Yersinia pseudotuberculosis*.The species of *Alcaligenes faecalis*, *Providencia vermicola* and *Enterobacter hormaechei* were identified in various stages of the vector and confirmed by biochemical and molecular techniques. It is found *Providencia rettgeri* proper candidate for paratransgenesis.

Introduction

Malaria is still is the important tropical diseases in the world. Malaria deaths were estimated 627000 worldwide in 2012. Based on last WHO report (2013), from 97 countries with ongoing malaria transmission, 12 are being in the pre-elimination phase, and 7 classified in the elimination phase (Organization, 2013). Based on Iranian Ministry of health report, 99% of malaria cases occur in Sistan and Baluchistan, Kerman and Hormozgan Provinces, Southeast of Iran. An estimated 33% of malaria cases were reported from Hormozgan, of whom 1450000 people were living in this area. Some socio-economic variables were increasing the number of

malaria cases in Pakistan and Afghanistan and migration of malaria cases to Iran. At the present, at estimated more than 1milion Afghans are registered as refugees in Iran(Organization, 2013).

The family Culicidae (Diptera) comprises at least 3531 species representing 112 genera divided into two subfamilies, Culicinae and Anophelinae (Harbach, 2013). According to the newest checklist of Iranian mosquitoes, 64 species representing seven genera occur in the country (Azari-Hamidian, 2007). Five *Anopheles* species was reported as the proven malaria vectors in southern Iran including; *An stephensi*, *An fluviatilis*, *An culicifacies*, *An dthali*, *An superpictus* and one as the suspected, *An pulcherrimus* (Nejati *et al.,* 2013) Naddaf *et al.,* (2010, 2012) reported the occurrences of the*An. fluviatilis* James species U and T in Southern Iran (Naddaf *et al.,* 2010; Naddaf *et al.,* 2012). Two distinct peaks of malaria transmission occur in the southern Iran : one in April-May and the other in September-October(Moosa-Kazemi *et al.,* 2014). The peak of malaria incidence coincides with the peak vectors density of *An. stephensi*, as the main vectors, whereas *An. fluviatilis* plays a secondary role in the malaria transmission (Moosa-Kazemi *et al.,* 2014). Indoor residual spraying (IRS) and distributed of long lasting impregnated bed nets are considered as the main strategy to control of malaria vectors in Iran during the malaria elimination phase (Nejati *et al.,* 2013). IRS may reduced the population of endophile species of *An. stephensi*, whereas this control method has bit effects on the population of *An fluviatilis* with exophilic behavior (Moosa-Kazemi *et al.,* 2007, Manouchehri *et al.,* 1976).

There are scattered studies about the bacteria flora in the vectors in Iran. A study on aerobic bacterial flora of *Periplaneta americana* showed that 12 species found inside the gut of cockroaches and reported the *Entrobacter cloacae* as proper paratransgenesis candidate (Akbari *et al.,* 2014). In the same study Chavshin *et al.,* (2012) reported 12 genera, and 40 species bacterial microflora in the midgut of the larvae, and 5 genera and 25 species in the midget of the adult form of *Anopheles stephensi* wild strain. *Pseudomonas* sp. was reported as the proper paratransgenesis candidate (Chavshin *et al.,* 2012). Only one research carried out on aerobic microbial community of sand fly *Phlebotomus papatasi* and the most prevalent isolates were *Proteus mirabilis* and *P. vulgaris* (Maleki-Ravasan *et al.,* 2013). The new tools was introduced to vector control considered as; genetic manipulation of microorganisms symbionts of insect stomach and also recombinant of selected genes which are affecting, limiting and stopped the parasite life cycle (Chavshin *et al.,* 2013). Paratransgenesis is new tool and original research to use it in an ongoing international research centers. One of the most basic and most important information for understanding paratransgenesis is identification of vectors gut microflora and sustainability status, location, and it is transferring situation of bacteria from larvae to adult stage.

Insect-borne microorganisms coexist in many aspects of life cycle such as nutrition, reproduction, resistance to environmental factors, maintaining and improving the immune system of the host, the protection strength of the mucosal barrier, metabolism, can play an important role in disease transmission.

Symbiont bacteria live in the mosquito stomach and may play an important role in development of the parasite, either enhance or inhibit it. In nature, mosquitoes feed on

different types of materials and are suitable host to infected them. Such as bacteria, fungi may be destroyed the parasites (Gwadz *et al.,* 1989; Lindh *et al.,* 2005).

Typical vector control measures such as indoor residual spraying may be influence on vector resistance as well as the harmful effects on the environment, human health (Pimentel *et al.,* 1992; Weill *et al.,* 2003).

Larvae of *An. fluviatilis* was reported in beneath rocks along river beds, under the exposed roots of trees, in crevices artificial pits, edges of swamps, lake margins. In water currents and water will mix with the plants (Eshghi *et al.,* 1976). Larvae was found in water and food that may be exposed to different types of microbes (Vatandoost *et al.,* 2004; Rodrigues *et al.,* 2010). Therefore, it is expected that the range of micro-organisms in the gut of larvae and pupae.

These microbes can be genetically engineered to express anti-parasite molecules and then again are added to larval habitats. This is a new method to control vector borne diseases is called paratransgenesis reducing pathogen transmission in an insect (Chavshin *et al.,* 2013). This method introduced successfully for some vectors such *Rhodnius prolixus* vectors of *Trypanosoma cruzi* and vector of Chagas disease, *Triatominae* spp and also *Glossina morsitans*, vector of African sleeping sickness (Aksoy *et al.,* 2008; Pontes and Dale, 2011).

This is a new method to control vector borne diseases is paratransgenesis, which could reduce or stop the pathogen transmission in a vector (Chavshin *et al.,* 2013) .This method introduced successfully for some vectors such *Rhodnius prolixus* vectors of *Trypanosoma cruzi* and vector of Chagas disease (Beard *et al.,* 2001), *Triatominae* spp and also *Glossina morsitans*, vector of African sleeping sickness(Aksoy *et al.,* 2008, Pontes and Dale, 2011). High diversity of the intestinal bacterial microbiota was registered using culture dependent in relation to different populations of *Phelebotomus argentipes* and *Lutzomyia longipalpis*. Thus it is thought that the variation in the nutritional behavior of mosquitoes, as well as the conditions that they encounter in their growth stages Bacterial micro-flow is affected and the overall capacity it can also affect the preservation and development of the malaria parasite (Chavshin *et al.,* 2012; Rajendran and Modi, 1981).

Materials and Methods

Area

This study was carried out in Hormozgan. In this area three villages Siahoo (E:56.13 N:27.47), Hormodar (E:58.17 N:28.14), and Fein (E:51.55 N:37.27) were selected (Fig. 1). Standard dipping method as well as pyrethrum space spray collection carried out in selected villages

Dissection

Larvae, pupae and adult stages were collected in selected villages and samples were transferred to entomology laboratory and were dissected as follow; the outer surface of each biological stage was sterilized in 70% ethanol for 1minute. A drop of normal saline (0.9%) was placed onto a glass slide mounted under the light microscope. A sample was transferred onto the prepared slide by stabbing the sample thorax with a needle-tip probe. While holding down the sample with the probe, the forceps were used to grasp the second to the last abdominal segment and gently pulled

off the sample abdomen in a single motion. The midget was remained with the immobilized thorax. The abdomen is discarded and using the forceps the mid gut from the thorax was detached. Midgut was inserted in the media under the lab hood. Holly body without mid gut was inserted in the standard media in the same standard condition.

Isolation of bacteria

The bacterial microflora of larvae, pupae and adult *An. fgluviatilis* through biochemical techniques was carried out using diagnostic test kits, API 20E and BHI. Bacteria were isolated in the following way. An Inside gastric tubes containing 5 ml of brain heart infusions (BHI) broth was placed on the vertex to homogeneous. Non-selective BHI broth was used as a culture medium for the growth of a wide range of microbes, especially bacteria. After 24 hours, samples were regarded as positive staining in BHI agar and again incubated overnight at 37°C. To obtain a single colony of bacteria, colonies with different phenotypes were subcultured and single colonies were examined for identification.

Identification of bacteria using biomedical test

Gram staining was performed using standard kit to separate bacteria morphologically. In addition, agar medium MacConkey was used to distinct. Biochemical characteristics of gram-negative bacteria were identified using traditional identification Gallery kit plus API 20 E media supported by bio merieux, France. The single and new colony of bacteria suspended as equal to 2 McFarland and then injected to kit hollows.

The samples were incubated for 24 hours at 37°C and after adding the standard reagents such as Kovics in NID after 2 min, Band A Barritts in VP after 10 minutes, Fel3 to TDA after 1 minutes, then results were analyzed by kit soft ware and recorded. Based on API kit protocol, the ranges of colors of API were various and the results were discussed. Results were analysied using API soft ware as instruments described by Biomerieux Company. Bacteria were identified at the level of the genus and sometimes species.

Identification of bacteria using molecular study

Fresh simple colonies incubated overnight in liquid cultures in nutrient agar were used to Genomic DNA extraction. DNA was extracted by boiling techniques. For genotyping, a 1500bp of 16s rRNA gene was amplified with specific primers as forward as 5'GAGTTTGATCTTGGCTCA 3' and Reverse as5' GTTACCTTGT TACGACTT3' that described by Weisburg protocol (Weisburg *et al.*, 1991). Amplification was carried out on the isolates with the following PCR cycling conditions: an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56.5°C for 40 s, and extension at 72°C for 40 s, and final extension at 72°C for 10 min (Karimian *et al.*, 2011).

DNA sequencing

The PCR products of the isolates were sequenced by Seqlab, Germany using the amplification primers. Consensus sequences obtained from forward and reverse sequences and their homologies with the available sequence data in GenBank were tested by using the basic local alignment search tool (BLASTn) alignment program and the NCBI nucleotide database NCBI (www.ncbi.nlm. nih.gov/BLAST).

Single colonies of bacteria isolated from the outer surface and the gut of three biological stages of *An. fluviatilis* collected from Sistan and Baluchistan Province was shown in Table1. Of the 746 single colonies were isolated from 40% of the gut samples and 60% of the outer surface of the species. The number of gut colonies in Dashtook village was found to be 37% and the lowest 28 % found in Uraky. Single colonies were growth on the outer surface, were found at least 24% in Uraky and maximum 42% in Dashtook Villages. Total single colony isolated from larvae, pupae and adults stages were found 79%, 12% and 9% respectively.

Table 2 was shown the biochemical reaction of API 20E kit of the bacteria were growth on the larvae, pupae and adults samples that were collected in three villages selected in Sistan-Baluchistan Province. This reaction was carried out after the exposure with 20 various sugars and amino acids.

In this reaction 12 species were identified including; *Providencia rettger, Morganella morganii, Enterobacter aerogenes, Pseudomonas oryzihabitans, Citrobacter braakii, Citrobacter freundii, Aeromonas hydrophila, Klebsiella oxytoca, Citrobacter koseri, Serratia fonticola, Enterobacter sakazakii* and *Yersinia pseudotuberculosis.*

Biochemical reaction of API 20E kit on bacteria was growth on the larvae, pupae and adults samples were collected in three villages selected in Hormozgan Province in 2013 (Table 2).

Results and Discussion

The result of the reaction of glucose and amino acids in bacteria samples, sometimes were various, so in this study the standard software for identification of the bacteria species was used. In addition, we found 12 species of bacteria from the gut and outer surface of *An. fluviatilis* and were recorded for the first time. We use non selective of BHI broth culture was used to improve the growth of a wide range of bacteria in the recent research. In this study the growth of almost all Gram-negative bacteria especially of the family of Enterobacteriaceae inside the BHI media.

The results of this study using the API 20 E showed that gut and surface of *An. fluviatili*s have a diverse community of aerobic and Gram-negative bacteria of the Enterobacteriacea.

However, many of these bacteria are variable and their presents depend on the environment or food supplies in the *Anopheles* larval habitats. A few non-transient bacteria (possibly coexist) isolated of the gut and outer surface of *An. fluviatilis* and were identified, which, most of them were belonging to genera of *Enterobacter* and *Citrobacter*. Although a large number of bacteria were decreased during the metamorphosis from larvae to adult stages (Moll *et al.*, 2001), only a small number of bacteria could transmission from larval to adult stages (trans-stadia forms).

Comparison of gut flora of larvae and adults showed that some *Provedencia* and *Morganella* species/strains occurrences in both stage the representative to trans-stadial condition. However, it is recommended that trans-stadial transmission of this species was examined using a phenotypic marker, such as a green fluorescent protein (GFP), which this method was used for *Asaia* bacteria previously (Favia *et al.*, 2007).

Table.1 Single colonies of bacteria isolated from the outer surface and the gut of *Anopheles fluviatilis* collected from Hormozgan Province, 2013

	Area	Larvae		Pupa		Adult		No	
		No	Percent	No	Percent	No	Percent	No	Percent
Single gram-negative colonies from Stomach of *Anopheles fluviatilis*	Hormozgan Province (Hormodar)	78	50	38	24.5	38	25.5	154	45
	Hormozgan Province(Fein)	37	40	30	32	26	28	93	27
	Hormozgan Province(Siaho)	75	80	5	5	14	15	94	28
Total		190	56	73	21	78	23	341	100
Single Gram-negative colonies from Exterior of *Anopheles fluviatilis*	Hormozgan Province(Hormodar)	263	82	33	10	25	8	321	57
	Hormozgan Province(Fein)	49	46	30	28	28	26	107	19
	Hormozgan Province(Siaho)	44	32	26	19	66	48	136	24
	Total	356	63	89	16	119	21	564	100
Total single colonies		546	60	162	18	197	22	905	55

Fig.1 Map of the study area, Hormozgan Province, Southern Iran

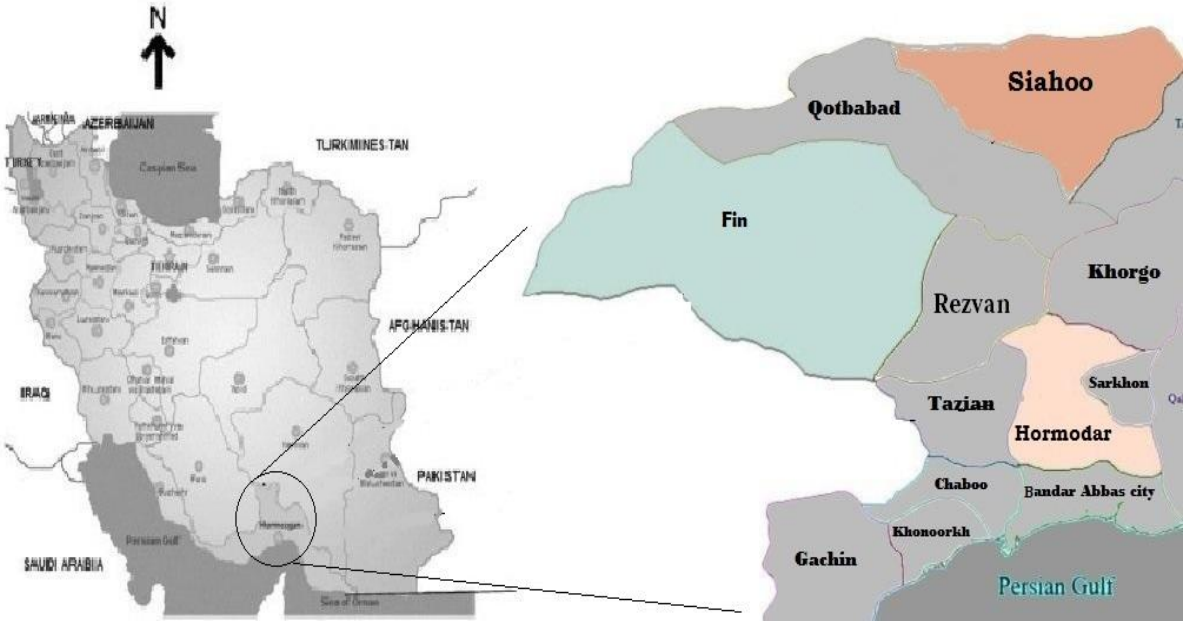

Table.2 Biochemical reaction of API 20E kit on bacteria were growth on the larvae, pupae and adults samples that were collected in three villages selected in Hormozgan Province, 2013

NO.	Test reaction	ResultsOf reaction														
1	ONPG	-	-	-	-	-	+	-	-	-	-	+	-	+	+	+	-	-	+	+	+	-
2	ADH	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-
3	LDC	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	+	-	-
4	ODC	-	-	+	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	-
5	CIT	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+	-	+	-
6	SH2	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
7	URE	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+
8	TDA	-	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-
9	IND	+	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-
10	VP	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	+	-
11	GEL	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-
12	GLU	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-
13	MAN	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+
14	INO	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	-	-
15	SOR	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	_	+	+	+	-
16	RHA	+	+	-	+	+	+	-	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+
17	SAC	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	+	_	-	-	+	-
18	MEL	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	+	+	+	+	-
19	AMY	+	-	+	+	+	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	+	+
20	ARA	-	-	-	-	+	-	-	-	-	+	+	-	+	+	+	-	-	+	+	+	+	-
Identified species		***Providencia rettgeri***						***Morganella morganii***			***Enterobacter aerogenes***		***Pseudomonas oryzihabitans***	***Citrobacter braakii***	***Citrobacter freundii***		***Aeromonashydrophila***		***Klebsiella oxytoca***	***Citrobacter koseri***	***Serratia fonticola***	***Enterobacter sakazakii***	***Yersinia pseudotuberculosis***

ONPG= ortho-nitrophenyl galactopyranosidase, **ADH**= arginine dihydrolase, **LDC** = lysine decarboxylase, **ODC**= ornithine decarboxylase, **CIT**= citrate, **URE**= urease, **IND**= indole, **VP**= Voges Proskauer, **GEL**= gelatinase, **GLU**= glucose, **MAN**= mannitol, **INO=** inositol, **SOR**= sorbitol, **RHA**= rhamnose, **SAC**= saccharose, **AMY**= amygdalin, **ARA**= arabinose, **TDA**=tryptophan deaminase, **MEL**=melibiose

Table.3 Isolated Bacteria of gut and outer surfaces of larvae, pupae, and adults stages of *An. fluviatilis* S.L. and their closest relative according to Blast in based on 0.7-1.3 kb of 16s rRNA gene, 2013

Genera and species of bacteria through biochemical	Stomach			outer surfaces			Topography			Genus and species Through molecular	Code and percent similarity of samples at Software blast and recorded in the gene bank	E Valve
	Adult	Pupa	Larvae	Adult	Pupa	Larvae	Mountain	Slope	Plain			
Providencia rettgeri	+	+	+	+	+	+	+	+	+	***Providencia rettgeri***	**GU457413.1 (%100)**	**0.0**
Morganella morganii	+	+	+	+	+	+	+	+	+	***Morganella morganii***	**KJ794191.1 (%100)**	**0.0**
Enterobacter aerogenes	+	_	+	_	+	+	_	+	_	***Enterobacter aerogenes***	**KJ997976.1 (%100)**	**0.0**
Pseudomonas oryzihabitans	+	_	+	_	+	+	_	+	+	***Alcaligenes faecalis***	**KJ672381.1 (%100)**	**0.0**
Citrobacter braakii	_	_	_	_	_	+	_	_	+	***Serratia fonticola***	**JN596121.1(%100)**	**0.0**
Citrobacter Freundii	_	_	_	+	_	+	_	+	_	***Citrobacter freundii***	**KM269033.1 (%100)**	**0.0**
Aeromonas hydrophila	_	_	+	_	_	_	_	_	+	***Aeromonas hydrophila***	**KM362733.1 (%100)**	**0.0**
Klebsiella Oxytoca	_	+	_	_	+	_	_	+	_	***Providencia rettger***	**KF534469.1 (%100)**	**0.0**
Citrobacter koseri/amalonaticus	_	_	+	+	_	_	+	_	+	***Providencia vermicola***	**KJ909024.1 (%99)**	**0.0**
Serratia fonticola	_	+	_	_	_	+	+	+	_	***Morganella morganii***	**LN558632.1(%100)**	**0.0**
Enterobacter Sakazakii	_	_	_	_	_	+	_	_	+	***Enterobacter hormaechei***	**KJ628424.1(%99)**	**0.0**
Yersinia pseudotuberculosis	+	+	+	_	_	+	+	+	_	***Providencia rettgeri***	**GU457413.1 (%98)**	**0.0**

Adult mosquitoes were targeted for paratransgenesis method (Lindh *et al.*, 2008). Trans-stadial characters were found among the bacteria in both Gram-negative and gram-positive (Wirth *et al.*, 1989). Load of the malaria parasite in infected mosquitoes by bacteria were reduced the prevalence of the *Plasmodium falciparum* and *P. vivax* (Gonzalez-Ceron *et al.*, 2003, Pumpuni *et al.*, 1996). In another study Gram-negative bacteria of the general of "Enterobacter" affect on *P. falciparum* (Cirimotich *et al.*, 2011).

In conclusion, It should be noted that, the wide range of the bacterial growth on the selective media and could be used for paratransgenesis and control of malaria in the future. Selected medium such as BHI, could be used to promote the growth of gram-negative bacteria, especially bacteria in the family Enterobacteriaceae. Simbiont

bacteria live in the mosquito gut and outer surface and could play an important role in development of the parasite, and the other hand cut the parasite life cycle. We were found *Providencia rettgeri*, *Morganella morganelli*, and *Yersinia psudotuberculosis* in biologic stages of *An. fluviatilis* s.l. which confirmed by biochemical and molecular techniques. We suggested *Providencia rettgeri* proper candidate for paratransgenesis.

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