DETECTION OF VIRULENCE GENES IN *AEROMONAS HYDROPHIL* ISOLATED FROM RABBITS BY POLYMERASE CHAIN REACTION.

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A B S T R A C T

The present study was performed on 260 rabbits (48 diseased and 212 freshly dead ones) from rabbit farms at Kaliobia Governorate for inspection of *Aeromonas hydrophila*. Samples were taken from these rabbits (liver; heart blood; lung; intestine; kidney and spleen from each one) after clinical and postmortem examinationfor bacteriological examination. The results revealed that, 195 *A. hydrophila* strains (12.5%) were isolated, 13(0.8%) from48 diseased rabbits and 182(11.7%) from 212 freshly dead ones. Moreover, higher rates of isolation of *A. hydrophila* from intestine (42.0%); liver (24.1%); heart blood (14.9%); lung (9.2%); kidney (6.7%) and spleen (3.1%).Ciprofloxacin, Enrofloxacin, Gentamicin, and Sulpha trimethoprim were the most proper antibiotics with the highest in vitro efficiency against isolated *A. hydrophila.* PCR results showed that, aerolysin (aerA) toxin virulence gene was detected in 10 out of 14 studied strains (71.4%). While haemolysin (hly) toxin virulence gene was detected in 4 out of 10 studied strains (40.0%).Moreover, the sequences obtained for aerolysin (aerA) gene had accession number KM 592977atGenBankand were 94% to 98% identical to the corresponding GenBank sequences. To the best of our knowledge, it may be the first record of studying the virulence genes of *A. hydrophila* strains isolated from rabbits in Egypt.

Keywords: *AEROMONAS HYDROPHIL,* RABBITS**,** PCR

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1. INTRODUCTION

Aeromonas hydrophila is a Gram-negative, rod-shaped, facultative anaerobic bacterium, oxidase and catalase positive. It had been reported in many countries in the world as normally inhabits in water sources and could be isolated from a wide range of mammals, birds and rabbits (Von and Zinterhofer, 1970; Glunder and Siegmann, 1989; Okewole et al., 1989; Efuntoye, 1995). *A. hydrophila* infection in rabbits causes hemorrhagic septicemia with a severe drop of hair, slight respiratory manifestations, enteritis, profuse watery diarrhea, emaciation and highly mortality rate (Paniagua et al., 1998; Kutkat et al., 2001). The pathogenicity of *A. hydrophila* is associated with the liberation of virulence factors and cell associated endotoxin. Virulence factors include the production of exotoxins (cytotoxin or enterotoxin); haemolysins; protease; aerolysin and ability to bind and to invade epithelial cells (Shaw and Hodder, 1978; Kozaki et al., 1989 ;Kirov et al., 1993; Krovacek et al., 1994; Aravena et al., 2014;Furmanek, 2014). The detection method of aerA was recently proposed as a reliable approach by which to identify a potential pathogenic Aeromonas strain by using methods involving PCR and restriction fragment length polymorphism analysis, the virulence genes of Aeromonas spp. were grouped as aerolysins - hemolysins, cytolytic enterotoxins, or cytotonic enterotoxins (Kaper et al.,1981) .

A PCR method for the amplification of the aerolysin gene was shown to detect a-hemolysin- positive *A. hydrophila* isolates from patients with diarrhea (Chopra et al., 1990).Very little information is available on Aeromonad virulence factors in rabbit strains of Aeromonas species isolated from rabbits. On the contrary, most of the research and epidemiological surveillance is centered on Aeromonas infections in fish. Therefore, the present study was planned for bacteriological characterization of rabbit *A. hydrophila* isolates and detection of some virulence genes of the isolated strains by using Polymerase Chain Reaction and make sequence to some virulence factors and submission them into Gene Bank.

2. 2. MATERIAL AND METHODS

2.1. Samples collection

A total of 260 rabbits of different ages and Sexes were examined in different rabbit farms at Kaliobia Governorate for bacteriological examination. Samples were taken from 48diseased rabbits and 212 freshly dead ones (liver; heart blood; lung; intestine; kidney and spleen from each rabbit) after clinical and postmortem examination. Each examined organ was taken alone in sterile plastic bag, kept in icebox and transferred with minimum delay to the laboratory for bacteriological examination.

2.2. Phenotypic identification and Phenotypic determination of virulence factors of A. hydrophila:

The surface of organs was seared by hot spatula, and then a sterilized loopfuls were inoculated onto tryptone soya broth and incubated aerobically at 37ºC for 24 hours. A loopful from incubated tryptone soya broth was streaked onto :tryptic soya agar ;MacConkey's agar;Aeromonas base agar; Rimler- shotts agar (R.S.) ; Thiosulphate – Citrate –Bile –Sucrose (T.C.B.S) agar; blood agar plus 10 mcg /liter ampicillin; starch agar; Eosin methylene blue agar (EMB)and milk agar media.All plates were incubated for 24hours at 37ºC.The developed colonies were picked up and subculture for purification. The purified colonies were morphologically identified by Gram stain and biochemical tests ((Quinn et al., 2002; Nicky, 2004, Songer and Post, 2005, Guadalupe Aguilera-Arreola et al., 2009 and Sendesh et al., 2011)

2.3. In-Vitro anti-microbial sensitivity test:

The isolated *A. Hydrophila* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method (Finegold and Martin, 1982).

2.4. Genotyping of Virulence genes of isolated A. hydrophila by PCR

PCR was applied by using two sets of primers for detection of two virulence genes that may play a role in virulence of *A. hydrophila*. These genes were aerolysin (aer A) and haemolysin (hly). It was applied on 14 random isolated *A. hydrophila* following QIAamp® DNA Mini Kitinstructions (Catalogue no. M501DP100); Emerald Amp GT PCRmastermix (Takara) with Code No. RR310Aand agarose gel electrophoreses (Sambrook et al., 1989).

2.5. Sequencing and phylogenetic analysis of aerolysin gene (Sanger et al., (1977)

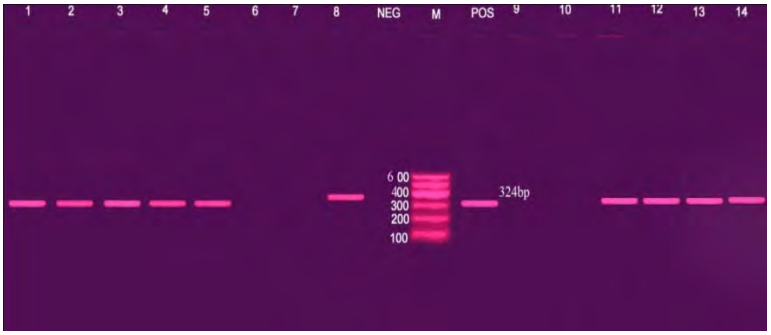
3. RESULTS

The clinical examination of studied rabbits showed clinical manifestations as anorexia, ruffed fur, depression, profuse watery diarrhea, a severe drop of hair and emaciation. Meanwhile, The postmortem lesions of freshen dead and scarified rabbits are signs of septicemia including congestion with petechial hemorrhages in internal organs as liver, lung, spleen, kidneys, heart and intestine showed sever enteritis, filled with watery fluid and distended with ganes. The results of *A. hydrophila* isolation (Table1) showed that 195 out of 1560samples (12.5%) were

positive for isolation, where 13isolates (0.8%) were isolated from 288 samples of 48 diseased rabbits and 182 isolates (11.7%) from 1272samples of212 freshly dead ones.

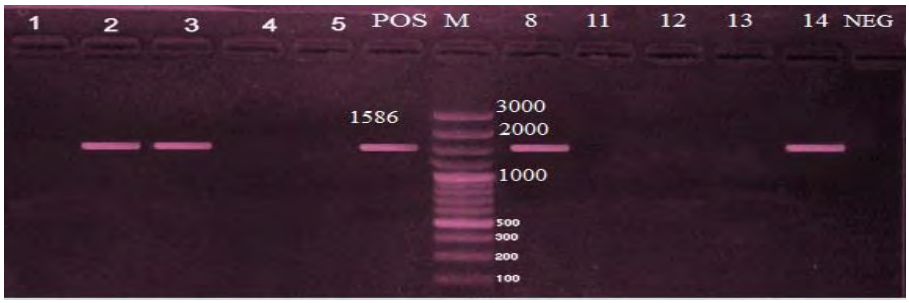
The bacteriological examination of studied organs revealed that, a total of 195 *A. hydrophila* strains were isolated, 82from intestine samples (42.0%) ; 47 from liver samples (24.1%);29 from heart blood samples (14.9%);18 from lung samples(9.2%) ; 13 from kidney samples (6.7%) and 6 from spleen(3.1%) as shown in Table (2) .The recovered isolates in this study grow well and showed white colonies on tryptone soya agar; pale colonies then become pink on MacConkey's agar media. While the same isolates showed large grayish circular, smooth, glistening colonies and most of them surround by beta haemolysis and newly isolated strain have a pungent foul odour on blood agar plus 10 ug /liter ampicillin; on Rimler- shotts medium (R.S) produced yellow convex colonies; on Aeromonas agar they give green colonies darker in center than emerging; they hydrolysis starch on starch agar and detected by logus iodine due to amylase enzyme;they give violet to metallic green sheen colonies on EMB media due to lactose utilization; they give yellow colonies on Thiosulphate –citrate –bile – sucrose (T.C.B.S) agar due to fermentation of sucrose and on the milk agar media detected protease enzyme was shown by the formation of a clear zone caused by casein degradation. The in- vitro sensitivity tests for isolated *A. hydrophila* (Table, 3) showed high sensitivity to Ciprofloxacin, Enrofloxacin, Gentamicin, and Sulpha trimethoprim meanwhile they were resistance to Penicillin, G., Ampicillin, Vancomycin, Oxacilline and Erythromycin.The results of virulence genes detection byPCR showed that aerA virulence gene was detected in 10 out of 14 studied strains (71.4%). While hly virulence gene was detected in 4 out of 10 studied strains (40.0%) as shown in Table (4).The aerA gene was amplified in 10 out of 14*A. hydrophila* strains giving product of 326 bp (photo, 1).The hly gene was amplified in 4 out of 10*A. hydrophila* strains giving product of 1586 bp (photo, 2).

Results of sequence of aerolysin of *A. hydrophila*: We have provided a Gene Bank accession number for our nucleotide sequence (Bankit 1760016Seq) is KM 592977. The sequences obtained were from 95 to 97% identical to the corresponding GenBank sequences (accession numbers AEF HM853019.1, and 96% with *A. veronii* bv. sobria aerA gene for aerolysin, AB109093.1, *A. hydrophila* strain Sb AY611033., *A. sobria* isolate S29-As aerolysin AF443393.1, *A. hydrophila* hemolysin gene, AF410466.1, *A.sobria* strain AS47- aerolysin JX293340.1 and 95% *A. sobria* partial aer gene for aerolysin protein, strain CB5869 AJ243046.1). sequence Distance and phylogenetic tree of this accession numbers in fig 1 and 2.



aerA

Photo (1): aerolysin (aerA) gene.Lane M: 100-600bpDNA Ladder. Neg.: Negative control. Pos.: Positive control (at 326bp). Lane 6; 7; 9 & 10: *A. hydrophila* (Negative). Lane 1; 2; 3; 4; 5; 8; 11; 12; 13 & 14: *A. hydrophila* (Positive).



hly

Photo (2): Haemolysin (hly) gene. Lane M: 100-3000bpDNA Ladder. Neg.: Negative control Pos.: Positive control (at 1586 bp). Lane 1, 4; 5, 11, 12 & 13: *A. hydrophila* (Negative). Lane 2; 3; 8&14: *A. hydrophila* (Positive).

Percent Identity

	1	2	3	4	5	6	7	8		
1		97.9	97.5	97.3	27.4	95.8	68.1	47.5	1	OUR
2	3.0		98.7	98.5	27.4	96.6	69.0	48.3	2	HM
3	4.0	1.6		98.5	27.2	96.6	69.6	47.7	3	ABI109093.1
4	4.3	2.0	2.3		27.4	96.8	69.0	47.9	4	AY
5	88.7	87.6	90.7	88.3		25.7	44.1	31.6	5	AF443393.1
6	4.6	3.0	3.3	2.9	95.6		68.8	47.3	6	AF410466
7	9.3	7.0	6.1	7.4	121.3	6.0		30.4	7	JX293392
8	128.6	122.0	127.3	123.9	4.0	133.3	114.7		8	AJ243046.1
	1	2	3	4	5	6	7	8		

Fig (1) sequance Distance

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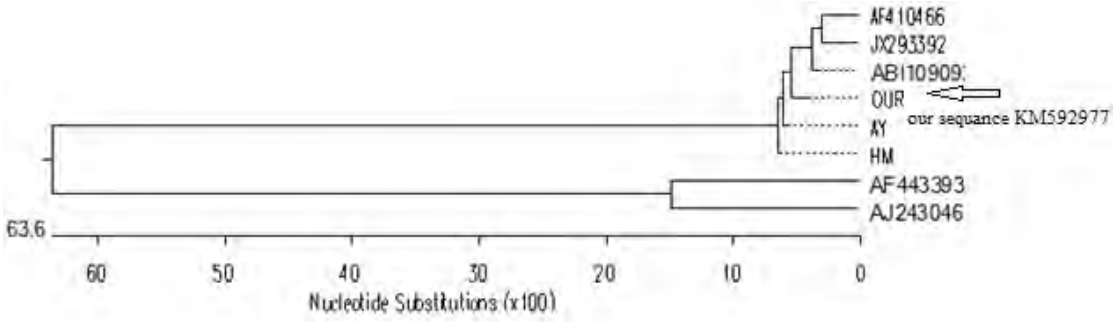

Fig (2) Phylogenetic relationships between the (aerA) gene sequence of the 8 strain and those members of an *A. hydrophila* complex. The construction was performed based on a 324 bp comparison using neighbor-joining method and the Mega program version 7.1.0 (44) our strain *A. hydrophila* (KM592977) has 97% of similarity of sequence with *A. hydrophila* strain AEF HM853019.1 ,and 96% with *A. veronii* bv. sobria aerA gene for aerolysin, AB109093.1, *A. hydrophila* strain Sb AY611033., *A. sobria* isolate S29-As aerolysin AF443393.1, *A. hydrophila* hemolysin gene, AF410466.1, *A. sobria* strain AS47- aerolysin JX293340.1 and 95% *A. sobria* partial aer gene for aerolysin protein, strain CB5869 AJ243046.1 was obtain

Table (1): Percentage of *A. hydrophila* isolated from studied rabbits

Rabbit case	Number of sample	Positive samples numbers	Positive percentage of *A. hydrophila*		
			%[1]	%[2]	%[3]
Diseased(48)	288	13	4.5	6.7	0.8
Freshly Dead(212)	1272	182	14.3	93.3	11.7
TOTAL(260)	1560	195	12.5	100.0	12.5

[1]Percentage in relation to total number of samples in each row
[2] Percentage in relation to total number of positive samples (195)
[3] Percentage in relation to total number of collected samples (1560)

Table (2): Total number and percentage of ***A. hydrophila*** isolated from different organs of studied rabbits' cases

Rabbit case		Number of rabbits	Positive Samples						Total			
			Liver	Heart Blood	Intestine	Kidney	Spleen	Lung				
			NO.	NO.	NO.	NO.	NO.	NO.	NO. of samples	NO. of Positive samples	Positive percentage %[1]	Positive percentage %[2]
Diseased		48	3	0	9	0	1	0	288	13	4.5	2.6
Freshly Dead		212	44	29	73	13	5	18	1272	182	14.3	35.7
TOTAL	NO.	260	47	29	82	13	6	18	1560	195	12.5	38.3
	%3	-	24.1	14.9	42.0	6.7	3.1	9.2	-	100.0	-	-

Table (3): In-Vitro anti-microbial Sensitivity test for isolated *A. hydrophila* strains

Antibacterial agent	Disc content	*Aeromonas hydrophila*
Ampicillin	10 ug	R
Ciprofloxacin	5ug	S
Enrofloxacin	10 ug	S
Erythromycin	15 ug	R
Norfloxacin	5ug	S
Gentamicin	10 ug	S
Doxycycline	30 ug	S
Penicillin G.	10 units	R
Sulpha trimethoprim	25 ug	R
Amoxicillin	30 ug	intermediate
Oxacilline	30ug	intermediate
Vancomycin	30 ug	R

N. B.: The beta lactamase antibiotics don't use in rabbit as it causes toxicity.

Table (4): The results of PCR amplifications of different used genes of *A.hydrophila*

Serial		Virulence genes	
		aerA	*hly*
	1	+	-
	2	+	+
	3	+	+
	4	+	-
	5	-	Not done
	6	+	-
	7	+	+
	8	+	-
	9	-	Not done
	10	-	Not done
	11	-	Not done
	12	+	-
	13	+	-
	14	+	+
Total	No.	10/14	4/10
	%	71.4	40.0

aerA (aerolysin), *hly* (haemolysin)

4. DISCUSSION

The infection with *A. hydrophila* is one of the most serious problems that causes diarrhea in humans, large animals, birds, rabbits and fish resulting in high economic losses due to the debilitating effect, which predisposes for many other diseases. Very little information is available on Aeromonad virulence factors in rabbit strains isolated from rabbits. Therefore, this study was planned for bacteriological characterization of rabbit *A. hydrophila* isolates and detection of some virulence genes in isolated strains. The results of clinical and postmortem examinations of studied rabbits were similar to that reported by (Efuntoye, 1995; Michael and Eckhaus 1997; Kutakat et al., 2001).The results of *A. hydrophila* isolation, (Table, 1) revealed that, 195 *A. hydrophila* strains (12.5%) were isolated, 13(0.8%) from48 diseased rabbits and 182(11.7%) from 212 freshly dead ones. These results came in accordance with that obtained by (Paniagua et al., 1998; Rodriguez-Calleja et al., (2006). Meanwhile, some reported higher incidence of*A. Hydrophila* isolation (Efuntoye, 1995; Abd El-Gwad and Abd El-Rahman, 2004). Moreover, higher rates of isolation of*A. hydrophila* from intestine (42.0%) ; liver (24.1%); heart blood (14.9%); lung (9.2%) ; kidney (6.7%) and spleen(3.1%) as shown in Table (2).Nearly similar results were recorded by (Paniagua et al.,1998 ; Rodriguez-Calleja et al., 2006). The morphological characteristics of the culture, Gram staining and the biochemical profile of *A. hydrophila* isolated was similar to those previously reported such as the fermentation of certain sugars or enzymatic reaction as amylase and lipase (Caper et al.,1981; Hsu et al .,1981; Carnahan et al .,1991; Sharon et al.,1992;Michael et al

.,1996; Quinn et al., 2002; Abbott et al., 2003; Sharon et al., 2003; Abd El-Gwad and Abd El-Rahman, 2004 ; Songer and Post, 2005; Jayavignesh et al., 2011).

The results of antibiotic sensitivity tests (Table, 3) revealed that, the isolated *A.hydrophila* strains were high sensitivity to Ciprofloxacin, Enrofloxacin, Gentamicin, and Sulpha trimethoprim meanwhile they were resistance to Penicillin, G., Ampicillin, Vancomycin, Oxacilline and Erythromycin. . Nearly similar results were recorded by (Mascher et al., 1988; El-Khashab and El-Yased, 2001; Yucel and Citak, 2003; Abd El-Gwad and Abd El-Rahman, 2004; Rogo et al., 2009;Jayavignesh et al., 2011). Our results disagreed with that recorded by (Davis et al., 1978; Fass and Barnishan, 1981; Redondo et al., 2004; Stojanov et al., 2010).

PCR results (Table, 4) showed that, aerolysin (aerA) toxin virulence gene was detected in 10 out of 14 studied strains (71.4%). While haemolysin (hly) toxin virulence gene was detected in 4 out of 10 studied strains (40.0%).The results of PCR for amplification of aerA gene in *A. hydrophila* isolates (photo, 1) showed that, the aerA gene was amplified in 10 out of 14 isolates giving product of 326 bp . Similar findings were recorded by (Hazen et al., 1978; Toranzo et al., 1986; Walke, 1997; Rabaan et al.,2001;Yousr et al., 2007; Kareem,2012; Aravena et al., 2014; Furmanek, 2014; Skwor, 2014). Meanwhile, the results of PCR for amplification of hly gene in *A. hydrophila* isolates revealed that, the hlygene was amplified in 4 out of 10 strains giving product of 1586 bp as shown in photo (2).Similar findings were recorded by (Rabaan et al., 2001; Yousr et al., 2007; Aravena et al., 2014; Furmanek, 2014; Skwor (2014). Moreover, the sequences obtained for aerolysin (aerA) of isolated *A.hydrophila* with provided Gene Bank accession number KM 592977were 94 to 98% identical to the corresponding GenBank sequences(AJ243047 for Filler et al., 2000; AB109093.1 for Song et al., 2004; HM853019.1 for Li et al. ,2010; JF298810.1 for Chan et al., 2010 and JX293341 for Das et al., 2012).

Finally from results of the present work we could concluded that; higher percentage of *A. hydrophila* infection was detected in rabbits. Ciprofloxacin, Enrofloxacin, Gentamicin, and Sulpha trimethoprim were the most proper antibiotics with the highest in vitro efficiency against isolated*A. hydrophila* can be used for treatment in cases of their infections. Also, PCR could indicate that the aerA virulence gene was detected in 10 out of 14*A. hydrophila* studied strains (71.4%), while hly virulence gene was detected in 4 out of 10 studied strains (40.0%). To the best of our knowledge, it may be the first record of studying the virulence genes of*A. hydrophila* strains isolated from rabbits in Egypt.

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الكشف عن بعض جينات الضراوة في ميكروب الايروموناس هيدروفيلا الممرض للأرانب بواسطة تفاعل البلمرة المتسلسل

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الملخص العربي

يعتبر ميكروب الايروموناس هيدروفيلامن أهم الميكروبات التي تسبب حالات الاسهال فى الانسان والحيوانات والارانب والتي تسبب خسائر اقتصادية كبيرةحيث انها تعتبر كعامل مساعد للاصابة بالميكروبات الاخرى. وبالرغم من ذلك فأنه نادرا ما تقوم الدراسات على هذا الميكروب والعوامل الممرضة فيه وعلاقته بالأرانب. وعلى ذلك فإن هذه الدراسة تلقى الضوء على هذه ا لميكروبات المعزولة من الأرانب و دراسة زراعتها على الأوساط الملائمة وكذلك الخصائص المورفلوجية و البيوكيميائية و عمل اختبارات الحساسية مع تحديد أهم الجينات الأكثر ضراوة بين العترات المعزولة و عمل تتابع نيوكليتيدى لبعض عناصر الضراوة و تسجيلها ببنك العترات . وقد أجريت هذه الدراسة على 260 أرنب (48 مريضة و 212 نافقة حديثا) وقد جمعت العينات من فئات الأرانب المختلفة من حيث العمر و الجنس من مزارع مختلفة بمحافظة القليوبية وأخذت العينات من الكبد و الرئة ودم القلب و الأمعاء و الكلى والطحال من كل حالة بعد اجراء الفحص الأكلينيكى و الصفة التشريحية. وقدأظهرت نتائج العزل لميكروبات الايروموناسهيدروفيلاتواجد195 معزولة من أجمالى 1560 عينة بنسبة 12.5% حيث كانت 13 عترة بنسبة 0.8% تم عزلها من الارانب المريضة بينما تم عزل 182 عترة بنسبة 11.7% من الارانب النافقة حديثا و سجلت اعلى معدلات للعزل من الاعضاء المختلفة كالاتى منالامعاءبنسبة 42.0% يليهاالكبدبنسبة 24.1 % واما القلبينسبة14.9 %والرئةبنسبة 9.2% والكلية بنسبة 6.7% وأخيراالطحال بنسبة 3.1%. أظهرت نتائج اختبارات الحساسية لعترات ميكروب الايروموناس هيدروفيلا انها شديدة الحساسية لكلا من السبروفلوكساسين و الاثروفلوكساسين و الجنتاميسين والسالف اترايميثوبريم و مقاومة للامبسلين والارثرومسين والبنسلين والفانكومايسين والاوكساسيلين ولقد أوضحت نتائج اختبار تفاعل البلمرة المتسلسل لجينات الضراوة (aerA, hly) لميكروب الايروموناس هيدروفيلا انه قد ثبت تواجد جينات الضراوة aerA بنسبة71.4% و 40.0% hly. ولقد تم عمل تتابع نيوكلتيدي لـaer A و تم تسجيله في بنك الجينات بالكود KM592977 .