Isolation and sequencing of insulin-like growth factor 1 (*IGF-1*) from Egyptian buffalo *via* RT-PCR

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ABSTRACT

Insulin-like growth factor 1 (IGF 1) is a member of a heterogeneous group of peptides with important growth-promoting effects in vitro as well as in vivo. It plays a fundamental role in postnatal mammalian growth as a major mediator through which growth hormone exerts its biological effects. We report the isolation and sequencing of full-length IGF-1 cDNA from bovine liver, encoding the IGF-1 protein. RT-PCR reaction was performed in which gene-specific primers corresponding to IGF-1 were utilised employing Pfu, a proof reading and high-fidelity thermostable DNA polymerase. A PCR product of 543 bp, including the open reading frame of IGF-1 gene corresponding to the expected theoretical product size, was successfully amplified. Sequencing of the purified PCR product and its double alignment against a previously known bovine gene identified it as a cDNA corresponding to IGF-1 with the open reading frame encoding the entire IGF-1 precursor of 154 amino acids.

Keywords: *Insulin-like growth factor 1, sequencing, Bovine IGF-1, RT-PCR.*

INTRODUCTION

Insulin-like growth factors (IGFs) are a group of structurally-related polypeptides that regulate the growth of many types of mammalian cells. Expression of mRNA transcripts for the IGF family has been detected in embryos of a variety of species. Therefore, several lines of evidence indicate that the polypeptide growth factors of the IGF family have an important role in early development both *in vivo* and *in vitro* (Jansen *et al.*, 1983). *In vivo*, Insulin-like growth factors are involved in several biological processes, such as growth, development and metabolism (Stewart and Rotwein, 1996). In addition, the culture media containing high concentrations of IGF-1 combined with estrous cow serum and granulose cells can improve the development of *in vitro*-produced embryos. Supplem-entation of culture medium with IGF-1 increased the proportion of human embryos developing to the blastocyst stage from 35% to 60% (Lighten *et al.*, 1998). In buffalo the addition of IGF-1 to the IVM medium stimulates oocyte maturation at a concent-ration of IGF-1 100 ng/ml (Pawsche *et al.*, 1998). While the addition of *IGF-1* to the bovine IVM medium (TCM199+ 10% FCS) did not affect the cleavage rate, but blastocyst yields were significantly increased at lower or higher concentration (50 or 500 ng/ml) of IGF-1 (Markkala and Makarevich, 2001).

Bovine IGF-1 is a 70-amino acid, basic, single chain polypeptide, with a molecular mass of 7649 Daltons. The bovine cDNA is 93 % identical to the human sequence, and the

amino acid sequence is 96% conserved (Fotsis *et al.*, 1990). Three disulfide bridges maintain the tertiary structure of the molecule (Watson *et al.*, 1999). Bovine, porcine and human *IGF-1* are identical. A single gene locus for *IGF-1* has been mapped to bovine chromosome 5 and the long arm of human chromosome 12 (Brissenden *et al.*, 1984). Both IGF-1 and IGF-II are made as secreted prohormones. Although similar in their biological function, they exhibit significant differences in their pattern of expression *in vivo*. In particular, IGF-1 is expressed in juvenile life and is almost exclusively synthesized in the liver under the control of growth hormone as predicted by the original hypothesis. IGF-II, by contrast, is expressed predominantly in the embryonic and foetal stages of mammalian development in a wide variety of different tissues. This suggests that the IGFs may have both paracrine and endocrine functions of intercellular communication in controlling the growth of many tissue types *in vivo* (Froesch *et al.*, 1985). In this concern, it was reported that liver is the main endocrine source of IGFs, but autocrine (within the cell)/paracrine (adjacent cells) activity is found in most tissues (O'Dell and Day, 1998). On the other side, IGFs may play a role in neonatal death and organ abnormalities of cloned cattle (Li *et al.*, 2007).

The aim of the present work was to isolate and identify *IGF-1*, an important gene from cattle involved in the regulation of reproductive functions.

MATERIALS AND METHODS

Collection of bovine liver samples and total RNA extraction

Bovine liver samples were collected from EL-Sharkawy Slauter, Shubra EL-Khima, Khalubia Governorate, Egypt. Liver tissues were taken from young (9- to 12-month-old) male hybrid brown Buffalo. Collected liver tissue was rapidly dissected into small pieces using sterile scalpel, immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA from bovine tissues was extracted using SV total RNA isolation kit (Promega) according to the manufacturer's procedure. All reagents were prepared in RNase-free water spiked with the autoclaved RNase-inhibitor diethyl pyrocarbonate (DEPC, 0.1%). Briefly, liver samples (30-50 mg) tissue were ground in small mortar and pestle using liquid N_2. The tissue was quantitatively transferred into sterile Eppendorf tube using 175 µl lysis buffer (cold). A volume of 350 µL SV RNA dilution buffer was added and mixed by inverting 3-4 times, then placed in a waterbath at 70°C for 3 min. Samples were centrifuged for 10 min at 12,000 rpm at 20°C and the cleared lysate was transferred to a fresh tube by pipetting. A volume of 200 µl 95% ethanol was added to the cleared lysate and mixed by pipetting 3-4 times. This mixture was transferred to the spin basket assembly and centrifuged at 12,000 rpm/ 20°C/ 1 min. The eluate was then discarded. A volume of 600 µl SV RNA wash solution was added, centrifuged at 12,000 rpm/ 20°C/ 1 min and the eluate was discarded. To remove any contaminating genomic DNA, 50 µl DNase mix were applied directly to the membrane inside the spin basket and incubated at room temperature for 15 min. SV DNase stop solution (200 µl) was added to the spin basket and centrifuged at 12,000 rpm/20°C/1min/. RNA wash solution (600 µl) was then added and centrifuged at 12,000 rpm / 1 min/ 20°C. Wash solution (250 µl) was added and the spin basket was centrifuged and transferred from the collection tube to the elution tube. Nuclease- free water (100 µl), i.e., DEPC-treated, was added to the membrane covering the surface of membrane, and then centrifuged. The yield of total RNA isolated from bovine

liver was spectrophotometrically determined at 230, 260 and 280 nm. The spin basket was removed and elution tube containing purified RNA was capped and stored at -70°C.

Reverse transcription and first-strand *IGF-1* cDNA synthesis

The reverse transcription (RT) reaction was carried out in a final volume of 20 µl. A master mix (MM) was used for reverse transcription to minimize pipetting errors (Table 1). It was prepared by the addition of reagents in 0.5 ml siliconized polyprobyl tubes. After brief centrifugation, MM was divided into 0.2 ml PCR tubes. Prior to use, total RNA from bovine tissue was thawed on ice, then denatured at 65°C for 15 min. Solutions and reagents of reverse transcription were thawed at 65°C for 30-60 sec, and then placed on ice throughout the preparation of the MM. Sterile tubes were used and also the pipette tips were changed after each pipetting. After the addition of 1 µg liver total RNA, pretreated with DNase RQ1 (Promega), to the reverse transcription reaction, the tube was mixed by gentle tapping, centrifuged briefly and then transferred to the PCR machine (9700 Perkin-Elmer). The RT reaction was carried out at 25°C for 10 min and 42°C for 1 hr followed by a denaturation step at 95°C for 5 min, and cooling to 4°C.

Polymerase chain reaction and gel electrophoresis

PCR reaction was carried out using a specific reaction mix (Table 2). Prior to use, cDNA from bovine tissue was thawed on ice. Then, the RT reaction was diluted 5, 10, 15 and 20 times and used directly for PCR. The reaction mix (50 µl) was performed in a thin-walled 200 µl PCR tubes containing cDNA template, 1X buffer, 200 µM dNTPs, 0.2 µM of each gene-specific primers IGF1-F and IGF1-R (Table 3), 1.5 units of high fidelity *Pfu* DNA polymerase and the final volume was made up to 50 µl with nuclease-free water (Table 2). Solutions and reagents of MM- were thawed at 65°C for 30-60 sec, mixed and then placed on ice throughout the preparation of the MM. After brief centrifugation, MM was divided into 0.2 ml PCR tubes, cDNA was added and the tubes were placed into thermocycler (9700PE). PCR was performed on cDNA template by employing a PCR program as follows: Initial denaturation step at 95°C for 5 min, then tubes were subjected to 35 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. As a negative control, tubes were prepared with water instead of cDNA template. Then PCR products were resolved by electrophoresis on 2% agarose gel in 1X TBE, stained with ethidium bromide and visualized with UV light of Gel Documentation System (Biometra Biomedizinische Analytik, GmbH), according to Sambrook *et al.* (1989).

DNA Sequencing

DNA sequencing was conducted by GATC Biotech (www.gatc-biotech.com, Germany) using Sanger Sequencing Technology on ABI Prism 3730XL (Applied Biosystems/Sanger) according to the dideoxy chain-termination method (Sanger *et al.*, 1977) The sequencing was carried out on purified *Pfu*-amplified PCR product using the sequence-specific sense primer IGF1-F (Table 3). The identity of the sequenced PCR product was examined using Blast Search against Genbank database of *Bos Taurus*. The pairwise alignment was performed with BioEdit Sequence Alignment Editor (Hall, 1999).

RESULTS AND DISCUSSION

In this work, we employed the power of the RT-PCR technique in order to produce a bovine *IGF-1* cDNA from Egyptian buffalos.

Harumi *et al.* (2001) have reported the cloning of a cDNA encoding a porcine type 1 insulin-like growth factor receptor (*IGF-1R*) *via* RT-PCR strategy. The sequence of a 4.2-kb product was determined and had an open reading frame encoding 1367 amino acids with 98.1 and 95.2% sequence similarity to the human and rat *IGF-1R*, respectively. Unlike thermostable polymerases classically used in the basic PCR process, the purity and integrity of RNA isolated from tissue or cultured cells are crucial to the success of RT-PCR. The spectrophotometric readings in the three wave lengths are summarised in Table 4. The samples were nearly free from proteins but many of them contain other impurities. However, a low A260/A230 ratio (lower than 1.8 – 2.2) may indicate guanidine contamination that can interfere with downstream processing. Therefore, in order to get rid of guanidine thiocyanate contamination, RNA was precipitated by adding NaCl to a final concentration of 0.1 M. In addition, the total conforming to standard prerequisites (Sambrook *et al.*, 1989) RNA prepared was of high quality as its two ribosomal bands were practically free of any degradation as judged by gel electrophoresis (data not shown).

Prior to PCR, the RT reaction containing synthesized cDNA was diluted several times (5x, 10x, 15x and 20x) and each dilution was independently used for the subsequent PCR step for the purpose of diluting out any possible contaminants, which could be present in the RT reaction that might interfere with thermostable DNA polymerase by inhibiting their activity (Joubés *et al.*, 1999). Standard PCR was also done to avoid false positives due to DNA contaminants (data not shown). Gene-specific primers, IGF1-F and IGF1-R (Table 3) were designed based on a previously cloned bovine *IGF-1* cDNA (Fotsis *et al.*, 1990) spanning the open reading frame with a theoretical PCR product of 543 bp. It is worth noting that we particularly used *Pfu* DNA polymerase as it possesses an intrinsic 3´-->5´ exonuclease activity that serves a proofreading function. It is a superior enzyme in applications such as cloning and protein expression requiring high fidelity PCR, because it exhibits the lowest error rate of any commercially available thermostable DNA polymerase (Flaman, 1994).

Agarose gel electrophoresis was subsequently performed in order to verify the product size which was resolved just above the DNA ladder band 500 bp, indicating a size that is slightly higher than 0.5 kb (Fig. 1). All RT dilutions (lanes 1-4) gave a specific single band at the abovementioned size. Several trials have been done and the obtained result shown in Fig. (1) is a representative and reproducible image. Then, the resulting PCR products were pooled and purified using Clean-up PCR purification kit (Promega). Subsequently, sequencing was carried out (GATC Biotech, Germany) in order to verify the identity of the PCR product. The sequence obtained was examined against previously known bovine sequences of *Bos Taurus* database GenBank using Blast search (www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9913). The results of this step confirmed that the sequence as expected is identical to a bovine *IGF-1* [EMBL accession no. X15726] (Fotsis *et al.*, 1990). The obtained GATC sequence together with the bovine *IGF-1* (Fotsis *et al.*, 1990) were analysed through a specialised alignment programme, (BioEdit Sequence Alignment Editor) as described by Hall (1999). The pairwise alignment results of the PCR product sequence against the bovine *IGF-1* cDNA (Fotsis *et al.*, 1999) is depicted in Fig. (2), showing that the sequence of the PCR product aligns perfectly with the *ORF* region of the bovine *IGF-1*.

The nucleotide and deduced amino acid sequences of the isolated *IGF-1* cDNA are

shown in Figure (3). Moreover, the mature IGF-1 amino acid sequence is also included (Fig. 3, underlined residues). In several organisms, it has been shown that the IGF-1 is synthesized as precursor protein and that formation of IGF-1 from this precursor requires proteolytic processing at both ends. The human *IGF-1* gene consists of six exons, five introns and at least two promoters (Rotwein, 1991). The human *IGF-1* coding region was found to be flanked by sequences encoding an amino-terminal peptide of at least 25 amino acid residues and a carboxyl-terminal peptide of 35 amino acids (Jansen *et al.*, 1983). Similarly, in relation to IGF-II, it was indicated that human IGF-II is synthesized initially as larger precursor molecules. The deduced preprohormone sequence contains a C-terminal propeptide of 89 amino acid residues, which was named E-peptide (Dull *et al.*, 1984). Moreover, work conducted on rat by Whitfield *et al.* (1984) indicates that pro-rIGF-II is synthesized as a 156 amino acid peptide precursor (17,619 Mr) containing mature rIGF-II 1-67 at its amino-terminus and an 89-residue carboxy-terminal peptide extension. Taken together, alternative splicing at the level of RNA we propose to be responsible for the complexity of the *IGF-1* gene expression. The human *IGF-1* gene was found to extend over at least 45 kilobase pairs containing five exons interrupted by four introns. The DNA sequence of exons 1 through 4 encodes the 195-amino acid precursor, while exons 1, 2, 3, and 5 code for the 153-residue peptide, confirming the hypothesis that at least two *IGF-1* mRNAs are generated by alternative RNA processing of the primary gene transcript (Rotwein *et al.*, 1986). The only exception for this generalisation has been described in chicken. The pattern of *IGF-1* gene expression appears to be simpler in chickens than in mammals, since a single predominant mRNA of 2.6 kb can be detected in liver polyadenylated RNA on northern blots (Kajimoto and Rotwein, 1989).

In conclusion, we have successfully isolated a bovine cDNA from liver tissues and subsequently confirmed the identity of the PCR product by DNA sequencing. This may provide a robust basis for its cloning in bacterial or prokaryotic expression vector where IGF-1 protein is possible to be mass produced. This may enable the testing of IGF-1 effects both *in vitro* and *in vivo*. *IGF-1* gene can then be introduced in a mammalian expression vector, cloned and used as a supplement in culture medium to increase embryo development.

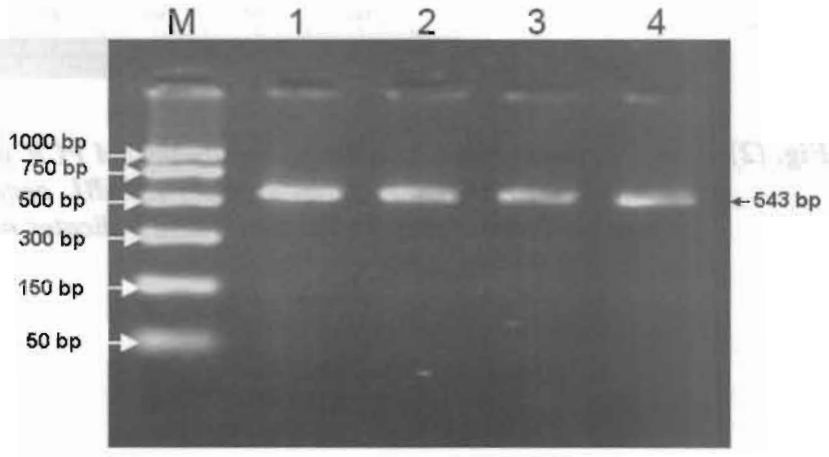

Fig. (1): Gel electrophoresis of RT-PCR products of IGF-1 of cattle livers. Lane M represents DNA ladder, Lanes 1-4 represent PCR product single bands of an approximate size of 543 bp corresponding to IGF-1 cDNA in which the RT reaction was diluted 5, 10, 15 and 20 times, respectively, prior to using in PCR.

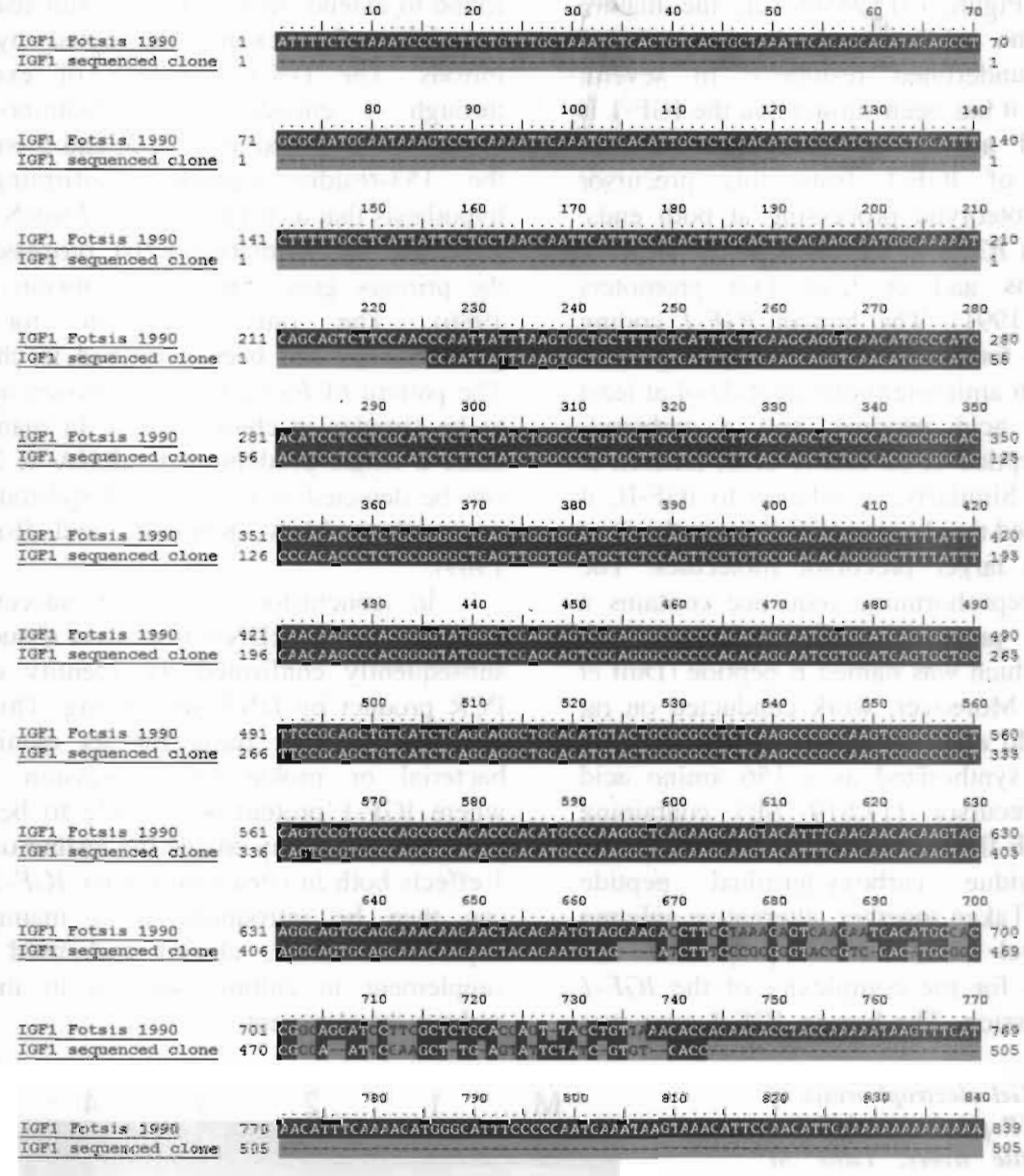

Fig. (2): Pairwise sequence alignment of the amplified PCR product (lower) against apreviously described bovine IGF-1 cDNA (upper) [EMBL accession no. X15726].Black shading indicates identical bases and grey shading indicates non-identical bases.

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1     ATG GGA AAA ATC AGC AGT CTT CCA ACC CAA TTA TTT AAG TGC TGC   45
1      M   G   K   I   S   S   L   P   T   Q   L   F   K   C   C   15

46    TTT TGT GAT TTC TTG AAG CAG GTG AAG ATG CCC ATC ACA TCC TCC   90
16     F   C   D   F   L   K   Q   V   K   M   P   I   T   S   S   30

91    TCG CAT CTC TTC TAT CTG GCC CTG TGC TTG CTC GCC TTC ACC AGC   135
31     S   H   L   F   Y   L   A   L   C   L   L   A   F   T   S   45

136   TCT GCC ACG GCG GGA CCC GAG ACC CTC TGC GGG GCT GAG TTG GTG   180
46     S   A   T   A   G   P   E   T   L   C   G   A   E   L   V   60

181   GAT GCT CTC CAG TTC GTG TGC GGA GAC AGG GGC TTT TAT TTC AAC   225
61     D   A   L   Q   F   V   C   G   D   R   G   F   Y   F   N   75

226   AAG CCC ACG GGG TAT GGC TCG AGC AGT CGG AGG GCG CCC CAG ACA   270
76     K   P   T   G   Y   G   S   S   S   R   R   A   P   Q   T   90

271   GGA ATC GTG GAT GAG TGC TGC TTC CGG AGC TGT GAT CTG AGG AGG   315
91     G   I   V   D   E   C   C   F   R   S   C   D   L   R   R   105

316   CTG GAG ATG TAC TGC GCG CCT CTC AAG CCC GCC AAG TCG GCC CGC   360
106    L   E   M   Y   C   A   P   L   K   P   A   K   S   A   R   120

361   TCA GTC CGT GCC CAG CGC CAC ACC GAC ATG CCC AAG GCT CAG AAG   405
121    S   V   R   A   Q   R   H   T   D   M   P   K   A   Q   K   135

406   GAA GTA CAT TTG AAG AAC ACA AGT AGA GGG AGT GCA GGA AAC AAG   450
136    E   V   H   L   K   N   T   S   R   G   S   A   G   N   K   150

451   AAC TAC AGA ATG TAG GAA GAC CTT CCT AAA GAG TGA AGA ATG ACA   495
151    N   Y   R   M   *                                         154

496   TGC CAC CGG CAG GAT CCT TCG CTC TGC ACG AGT TAC CTG TTA AAC   540

541   ACC                                                         543
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Fig. (3): Nucleotide and deduced amino acid sequences of bovine IGF1 cDNA; generated with BioEdit Sequence Alignment Editor (Hall, 1999). Bold underlined sequences denote sense (IGF-1F) and antisense (IGF-1R) primers used for RT-PCR. The codon usage was optimised for mammalians; start codon = ATG and stop codon = TAG (denoted by an * asterisk). The total sequence corresponds to an ORF of 543 bp encoding 154 aa. The mature IGF-1 protein is outlined.

Table (1): Composition of the master mix for reverse transcription reaction.

Reaction component	Concentration	Volume (ul)
10x Reaction Buffer	100 mM Tris, 500 mM KCl; pH 8.3	2.0ul
25mM Mg Cl_2	25 mM	4.0ul
dNTPs	dATP, dCTP, dTTP, dGTP;10 mM each	2.0ul
Primer (Oligo-p$(dt)_{15}$	0.02 A_{260} units/μl (0.8 μg/μl)	2.0ul
RNase Inhibitor	50 units/μl	1.0ul
AMV Reverse Transcriptase		0.8ul
Gelatin	0.5 mg/ml (0.05% [w/v])	1.0ul
Sterile water	-	3.2ul
RNA sample	(0.25ug/ul)	4.ul
Total		**20ul**

Table (2): PCR master mix reaction.

Component	Concentration	Vol. (µl)	Final concentration
Pfu DNA Polymerase 10X Buffer	200mM Tris-HCl (pH 8.8 at 25°C), 100mM KCl, 100mM (NH4)2SO4, 20mM MgSO4, 1mg/ml nuclease-free BSA, 1% Triton® X-100	5µl	1X
dNTP's mix	10 mM each	1µl	200µM each
IGF1_R	10µM	1µl	0.2 µM
IGF1_R	10µM	1µl	0.2 µM
cDNA template*	-	2µl#	<0.5µg/50 µl
Pfu DNA Polymerase	3u/µl	1µl	1.5u/50µl
Nuclease-free water	-	39µl	-
Total		**50µl**	

* The RT reaction was diluted 5X, 10X, 15X and 20X. The diluted RT reaction was subsequently used for PCR.
The cDNA template from the RT reaction was excluded from the negative control reaction.

Table (3): Primers used for PCR amplification of Bovine IGF-1.

Primers	Primer sequences (and positions)	Length	T_m(°C)
Forward	*IGF1-F 5'-ATGGGAAAAATCAGCAGTCTTC-3'(1-22)*	*22 bp*	*62*
Reverse	*IGF1-R 5'-GGTGTTTAACAGGTAACTCGTG-3' (543-565)*	*22 bp*	*64*

EMBL accession number for the bovine IGF-1 sequence, on which primer design was based, is [X15726].
The annealing temperature actually used for the PCR reaction was 57°C.

Table (4): Spectrophotometric absorption of extracted RNA before and after purification.

Total RNA Samples	$O.D_{260}$	$O.D_{280}$	$O.D_{230}$	$\lambda_{260}/\lambda_{280}$	$\lambda_{260}/\lambda_{230}$
Before NaCl treatment	0.067	0.041	0.058	1.65	1.16
After NaCl treatment	0.108	0.058	0.063	1.93	1.71

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الملخص العربي

فصل واجراء تسلسل لعامل النمو شبيه الإنسيولين -١ من البقر المصرى باستخدام تفاعل البلمرة العكسى

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** قسم الكيمياء الحيوية بكلية الزراعة جامعة القاهرة – جيزة – القاهرة

يعتبر عامل النمو شبيه الإنسيولين ١ أحد أفراد مجموعة من الببتيدات التى لها دور فعال فى تنشيط النمو والاخصاب خصوصا فى الثدييات قبل وبعد الولادة حيث يعمل كهرمون والذى يفرز أساسا من الكبد . تركز الإهتمام فى هذا العمل على دراسة العامل المذكور بغية الاستفادة منه فى رفع خصوبة الجاموس المصرى حيث تركزت الدراسة على التعرف على الجين المسؤل عن هذا العامل عن طريق فصل الحمض النووى الريبوزى الكلى RNA من كبد البقر المصرى كعضو أساسى لتواجد هذا العامل ثم إجراء عملية النسخ العكسى reverse transcription له بغية الحصول على التسلسل المقابل من الحامض النووى الدىأوكسى ريبوزى cDNA باستخدام تقنية RT-PCR وباختيار البادئات المتخصصة لهذا الجين.وأظهرت الدراسة أن الشظية الناتجة من تفاعل البلمرة المتسلسل ذات حجم قدره ٥٤٣ زوج من النوتيدات وبعد تنقية هذه الشظية تم عمل تسلسل نيوتيدى لها حيث أظهرت النتائج تطابق هذا التسلسل مع جزء كبير من تسلسل الجين الحقيقى لعامل النمو شبيه الإنسيولين-١ .