Identification and expression of β-defensin genes in Egyptian buffalo (*Bubalus bubalis* L.) infected with mastitis disease

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ABSTRACT

Mastitis is a disease that causes an inflammation of mammary gland (MG). The ability of animal's immune defense system to cope with infections of MG still are unknown .The β- defensin protein molecules of the innate system that was expressed by β- defensin gene may be candidate genes for resistance to mastitis. Therefore, the present investigation was aimed to isolate these genes from infected lactated female animals and amplify them using PCR in the presence of specific β- defensin primers, examine whether mastitis activates the innate immune system or not as indicated by using DD-PCR technique . Results indicated that the amplified PCR products were 2060 and 410 bp for healthy (control) and infected animals, respectively which were digested by Hinf1 and pvuII restriction enzymes for genotyping. Four fragments (150, 410, 600 and 900 bp) were produced from digestion of 2060 bp ,whereas 410 bp was digested to 180, 130 and 100 bp fragments that was different from healthy animals and could be associated with infection. The results of DD-PCR revealed several bands that were only expressed in the infected animals such as cDNA 2100 bp defense fragment which clearly appeared with presence of specific defense primer (forward).

Key words: *Mastitis, Mammary gland (MG), β- defensin gene, DD-PCR technique.*

INTRODUCTION

Innate immunity of animal produces antimicrobial peptides (AMP) after a challenge by pathogens; these peptides are named defensin proteins which display an important role against antimicrobial activity. Mammalian defensins protein are classified into α, β, and θ sub-classes (Boman, 2003). The major sub classes of defensins family is β-defensins which act as signaling molecules in connecting between innate and adaptive immune responses in higher organisms (Yang *et al.*, 2001) . β-defensins genes code multifunctional of peptides with a broad range of antimicrobial activity (Roosen *et al.*, 2004 and Rainard and Riollet, 2006). In most cases; their coding sequences consist of 2 exons. The first exon includes the 5′-untranslated region and the leader domain of the preprotein; the second exon encodes the mature peptide with the 6-cysteine domain (Choi *et al.*, 2012).

The Egyptian water buffalo (*Bubalus bubalis, L*) is the river buffalo that belongs to Mediterranean buffalo (Elbeltagy *et al.*, 2008). It becomes settled in Egypt for hundreds of years and has become the most important domestic animal for meat and milk production (Hafez *et al.*, 2008). Buffalo remains poorly

understood in term of innate immunity (Das *et al.*, 2006) although; it is known to be relatively more resistant to mastitis than other species (Joshi and Gokhale, 2006).

Mastitis is a complicated disease which caused by wide range of microorganisms such as *Staphylococcus aureus* and *Streptococcus agalactiae.* It is described as an inflammation of the mammary gland (MG) of animal that develops as a response to pathogenic microorganisms entering through the teat canal and multiplying inside the gland(Bannerman *et al* ., 2004 and Rainard and Riollet, 2006). In dairy cattle, mastitis is the most costly disease due to clinical mastitis includes veterinary services and cost of treatment, reduced milk production in the remaining lactation, milk disposal due to antibiotic treatment, early execution, extra labor and decreased milk quality. The reasons for the differences of pathogen in the ability of the host's immune defense system to cope with infections of MG of animal are unknown but, the β- defensin peptides of the innate system are known to display some pathogen specificity (Jia *et al.*, 2001). It was hypothesized that β - defensin genes may be candidate genes for resistance to mastitis disease (Pawlik *et* al., 2009). Therefore, the present investigation was aimed to isolate and amplify β - defensin genes from infected lactated female animals with mastitis using PCR in the presence of specific β defensin primers as well as expression of β-defensin *m*-RNA transcript in infected animals was studied through differential display PCR (DD-PCR) in the presence of a specific defensing primer (forward) and three random primers.

MATERIALS AND METHODS

Animals

Ten lactating female Egyptian buffaloes showing the symptoms of mastitis disease (Swelling, and redness of the udder , reduced and altered milk secretion from the affected quarters accompanied by fever, and anorexia of the animal) were precisely selected from the Faculty of Veterinary Medicine farm (Edfina ,Beheira governate) and Faculty of Agriculture farm (Ibis) Alexandria University in addition to six healthy female Egyptian buffaloes (heifers) randomly chosen from the previous places and studied as a negative control in the present investigation.

Samples collection

Blood samples were pulled from Jugular vein of animals by an authorized veterinarian and collected in coated tubes with K3EDTA then, stored at – 20 °C for DNA extraction.

DNA extraction and Amplification of defensin genes using specific primers

Total genomic DNA was isolated from the whole blood samples using DNA purification kit according to the manufacturer´s instructions (GF-1 – vivants, USA)**.** β-Defensin gene of the examined animals was amplified in the presence of specific defensin primers with sequences of Ryniewicz *et al.*, (2003):5' - GCCAGCATGAGGCTCCAT -3' (forward) and 5' – AACAGGTGCCAATCTGT -3'(reverse). PCR was performed in a reaction volume of 25 µl using 25 ng genomic DNA of each sample, 25 pmol of each primer, 10X Taq DNA polymerase buffer including MgCl2, 0.2 mM dNTPs and 5 unit/ µl Taq DNA polymerase (Promega ,USA). Initial denaturation for 5 min at 95°C was followed by 34 cycles of 1 min at 94°C, 45s at 63°C annealing temperature , 1 min at 72°C for polymerization and a 10 min final extension at72°C. The amplified DNA products were

electrophoresd on 2% agarose gel (GibcoBRL), stained with ethidium bromide, visualized on a UV Transilluminator and photographed by gel documentation system (Alpha-chem Imager, USA) .

Genotyping for the segment of the defensin genes using RFLP

The amplified fragment of defensin genes from healthy and infected animals cut and eluted individually from the agarose gel for digestion using restriction enzymes, *Hinf1* and *PvuII*. However, 5 µl of PCR product was added to a PCR tube containing 2.5 µl buffer (10X), 1 µl for each enzyme and 16.5 µl distilled sterile water .The total volume reaction of 25 µl was put at 37°C for 2 h to digest fragments and left for 10 min then heated at 75°C for killing enzyme and kept at 4°C. DNA fragments were separated on 2% agarose gel, stained with Ethidium bromide, visualized by UV Transilluminator and photographed.

Differential display -PCR (DD -PCR)

DD -PCR was performed as described by Chin *et al.,* (2000). Total RNA was extracted from blood sample of animals using GStract^{TM} RNA isolation kit II according to manufacturer's instructions (Maxim Biotech INC, USA). The cDNA was generated using the ExpandTM Reverse Transcriptase (Boehringer, Mannheim, Germany). Four primers were used for each cDNA sample, one of them was specific for β- defensin gene as described by Ryniewicz *et al.,* (2003): 5' -GCCAGCATGAGGCTCCAT-3' (forward) and the others primers were randomly chosen as Cellulase2(5'GAYGARCANGARCAYMG-3'),Cellulase3(5'GGYGGYTGGAATGARGG-3')andChitinase15**(**5'GGYGGYTGGAATGARGG-3'). The total reaction mixture was 25 µl containing 1 µl of 10 pmol of each primer, 2.5 µl of 0.2Mm dNTPs, 2.5 µl of 5mM MgCl2, 5 µl of 1 x PCR reaction buffer, 7.3 µl sterile distilled water and 1.5 µl cDNA template. The amplification program was one cycle at 95°C for 5 min (hot start) followed by 40 cycles at 95°C for 30 sec, 1 min at 30-32°C for annealing, 1 min at 72°C for extension , 10 min at 72°C for final extension . PCR product were separated on 1.5% agarose gel, stained with Ethidium bromide, visualized and photographed.

RESULTS

PCR amplification and Genotyping of β-defensin gene using RFLP

The specific primers defensin succeeded to yield one band with molecular weight 2060 bp in healthy animals (control) and 410 bp in infected animals (Fig.1 and 2) . Restriction enzymes (*Hinf1* and *PvuII*) were able to digest the PCR product where, 2060 bp fragment was digested to 150, 410, 600 and 900bp fragments in control while , 410 bp was digested to three fragments as 180, 130 and 100 bp in infected animals as shown in Fig. (3 and 4).

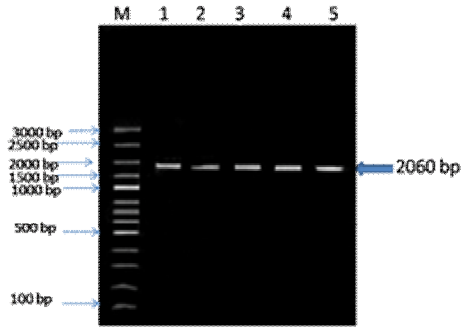

Fig. (1): 2% agarosegel eiectrophoresis of PCR products generated by the Beta-defensin gene primer. Where M: DNA marker (1000 -100 bp) Lanes 1-10 represent infected animals.

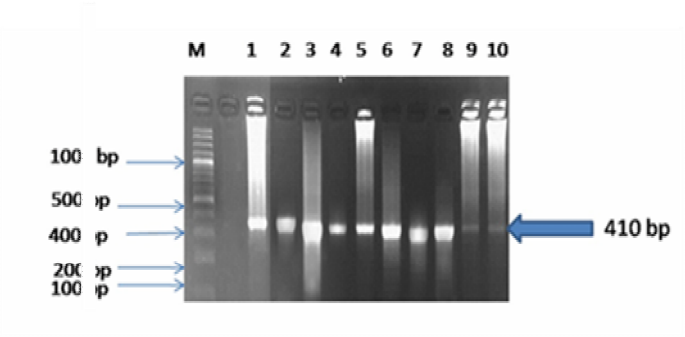

Fig. (2): 2% agarosegel eiectrophoresis of PCR products generated by the Beta- defensin gene primer. Where M: DNA marker [3000-100 bp}, Lanes 1-5 represent healthy animals. (control).

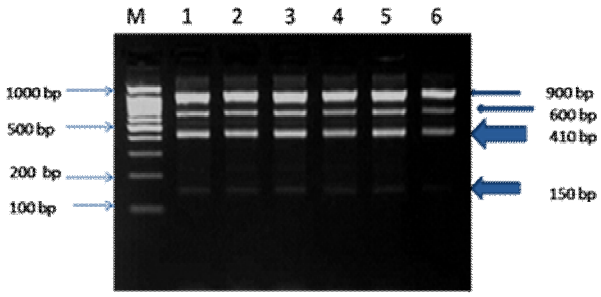

Fig. (3): The PCR fragment (2060 bp) of healthy Egyptian buffalos (control) digested by Hinfl and PvuII Where, M; DNA marker (1000 - 100 bp) and 1- 6 lanes samples.

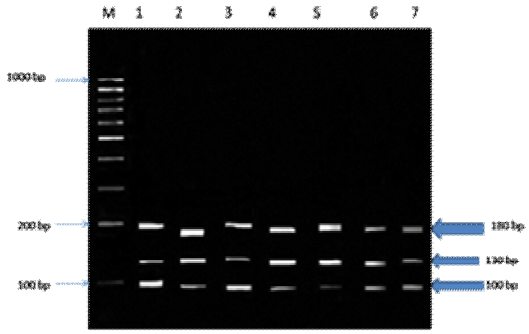

Fig. (4): The PCR fragment (410 bp) of infected animals (Lactating Egyptian buffalos) digested by Hinfl and Pvull. Where, M : DNA marker (1000 -100 bp) and 1-7 lanes samples .

Differential display PCR analysis (DD-PCR)

The results of expression of β-defensin m RNA transcript using differential display PCR technique in the presence of Cellulase2, Cellulase3, Chitinase 15 random primers and a specific defensin primer (forward) revealed that several common bands were expressed in healthy and infected animals but, some bands were only appeared in the infected animals such as 1500bp and less than 500bp bands which was produced in the presence of cellulase 2 primer (Fig.5A) also, The bands with lengths of 1000bp and less than500 bp that were clearly displayed in the presence of cellulase 3 primer and 1500 bp and 200bp bands also were shown with Chitinase 15 primer (Fig.5B and 5C). Specific defensin primer (forward) succeeded to express only the bands of 2100, 1000 and 800 bp in size in the infected animals at the same time, this primer clearly yielded bands as 1500 and less than 1000bp that was expressed in healthy animals (control) as shown in Fig. (5 D).

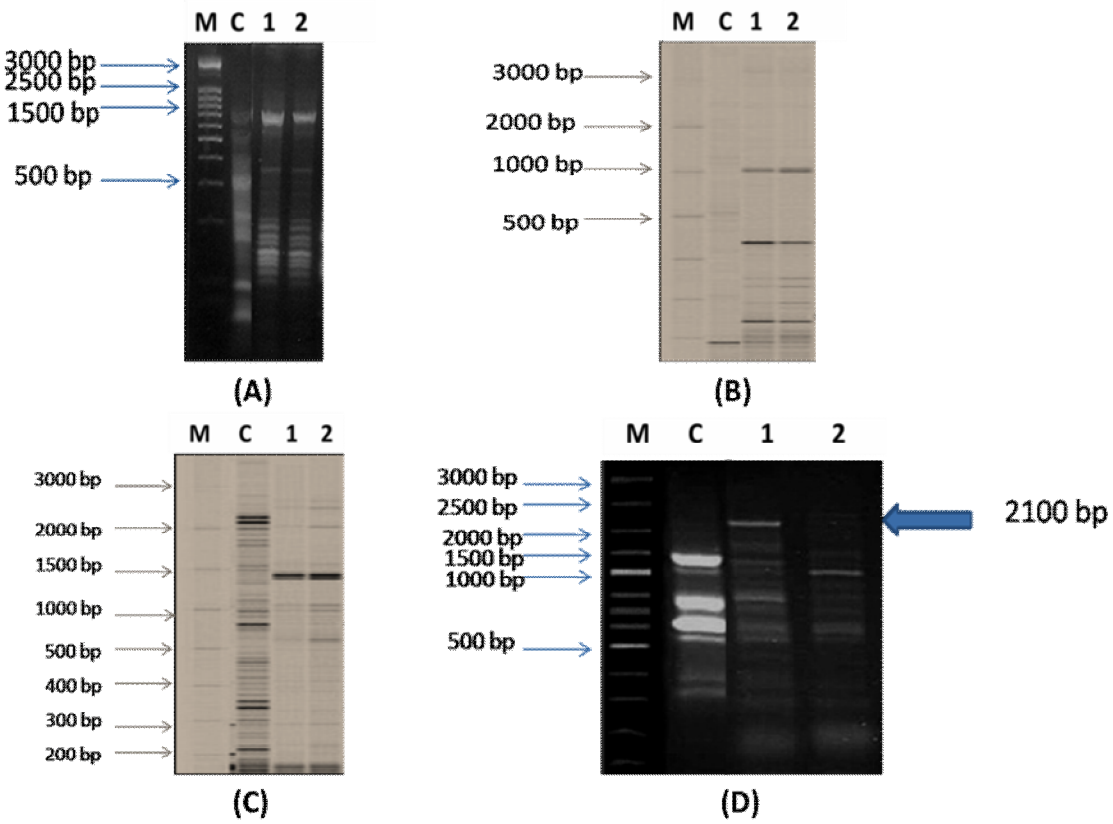

Fig . (5): Differential display (DD-PCR) with cellulase 2 (A), cellulase 3(B) ,chitinase 15 primers (C) and specific defensin primer forward (D), respectively where ,M: DNA ladder , lane (C) represents healthy animals (Control) and lanes (1-2) infected animals.

DISCUSSION

Based on the present results, it was found that specific defensin primers succeeded to amplify (2060 and 410 bp) fragment of β-defensin genes in both healthy (control) and infected animals. Ryniewicz *et al.,* (2003) and Hafez *et al.,* (2008) used the same defensin primers to amplify β- defensin genes of lactating cows animals and the last study was succeeded to differentiate between the β-defensin genes of the Egyptian and Frezian cows where, two major PCR products 1638 bp and 429 bp were amplified in Frisian while, only one band 429 bp in length was obtained in Egyptian cows, as a result of RFLP in our investigation. Both *Hinf1* and *PvuII* were able to digest the PCR products of healthy and infected animals resulting non identical pattern fragments and this may refer to the existence of genetic variations between healthy and infected animals or genetic polymorphism that may be as a result of infection .Results obtained in this study are in accordance with results performed by Chang-Hong *et al.,* (2008) whereas, the PCR products of the promoter of cow lactoferrin gene was digested by *Hinf1* enzyme. Variation was observed in RFLP banding patterns between healthy and infected cow animals with mastitis suggesting

that this polymorphism might be a mutation that could be associated with mastitis susceptibility.

Moreover, the mRNA differential display method was applied to identify the DNA expression affected by the mastitis. The results showed different expression patterns that were observed as bands present in infected animals compared with control. These bands may be one of these defensin genes that up-regulated due to the infection of animals which caused increasing in the level of mRNA. Our results also are in correspondence with the results that was obtained by Goldammer *et al.*, (2004) who reported that inflammation in the mammary epithelial cell of cows by Staphylococcal mastitis disease significantly increased the levels of mammary mRNA expression of β-defensin 5, suggest significant contribution of the innate immune system to counteract to mastitis. However, Swanson *et al.*, (2004) found that lingual antimicrobial peptide (LAP) mRNA expression is located abundantly in the ductal linings of the teat and cistern of mastitic quarters of animals. This means that LAP mRNA expression is induced at the site of infection and could also play an important role in regulatory pathways in mammary gland development, milk production and resistance or susceptibility to mastitis (Silveri *et a*l., 2006 and Das *et al.*, 2010).

Based on the finding results in the present study, it can conclude that further studies will be required to identify functional differences or specificity for β-defensin genes ,also, a better understanding of the roles of these defensin genes that could be useful a good markers of mastitis for improving general health or resistance to microbial infections in farm animals.

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الملخص العربي

التعبير الجينى لجينات بيتا الدفاعية فى الجاموس المصرى المصاب بمرض حمى التهاب الضرع

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يسبب مرض حمى التهاب الضرع التهاب فى الغدد اللبنية لضرع الحيوان . وقدرة مقاومة الجهاز المناعى لعدوى الغدد اللبنية للحيوان غير معروفة . ووجد ان جزيئات بروتينية من النوع بيتا واتى يفرزها الجهاز المناعى للحيوان من خلال جينات بيتا الدفاعية قد تشارك فى مقاومة الحيوان لهذا المرض اثناء العدوى .لذلك هدفت الدراسة الحالية الى عزل هذه الجينات من حيوانات اناث حلابة مصابة بالمرض من الجاموس المصرى بالاضافة الى جاموس سليم للمقارنة (كنترول) .ولتحقيق هذا الهدف تم استخدام تكنيك تفاعل البلمرة المتساسل فى وجود بادئات متخصصة من النوع بيتا(beta defensin genes) لتكبير جينات بيتا الدفاعية . وايضا تم دراسة التعبير الجينى لجينات بيتا الدفاعية على مستوى ال cDNA فى كلا من الحيوانات المصابة والكنترول باستخدام differential display . اظهرت نتائج تفاعل البلمرة المتساسل ان طول جين بيتا الدفاعى ٢٠٦٠ زوج من النيكلوتيدات فى الكنترول،٤١٠ زوج من النيكلوتيدات فى المصابة وكانت نتائج هضم جينات بيتا الدفاعية باستخدام تكنيك RFLP بانزيمات القطع *Hinf1 & PvuII* ان هناك اختلاف فى أطوال النيوكلوتيدات الناتجة من الهضم بين الحيوانات المصابة والسليمة تحت الدراسة فكانت اربع شظايا ١٥٠، ٤١٠، ٦٠٠، ٩٠٠ زوج من النيكلوتيدات فى الكنترول بينما اعطت ثلاث شظايا ١٨٠، ١٣٠ ١٠٠ زوج من انيكلوتيدات فى المصابة وربما هذا الاختلاف بسبب العدوى. وأظهرت نتائج استخدام تقنية ال differential display على مستوى ال cDNA أن هناك بعض الجينات الدفاعية من النوع بيتا حدث لها زيادة نشاط (up-regulated genes) وجينات أخرى حدث لها تثبيط (down-regulated genes) ومن الامثلة الشظية التى ظهرت بطول٢١٠٠ زوج من النيكلوتيدات مع البادىء بيتا الدفاعى (forward) فى المصابة ولم تظهر مع الحيوانات السليمة (الكنترول).