



## Virulence Potential of *Penicillium Chrysogenum* Isolated from Subclinical Bovine Mastitis

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### Abstract

The present study aimed to the isolation and identification of *Penicillium chrysogenum* from subclinical bovine mastitis as well as the evaluation of their potential to produce the main virulence factors by assessing proteinase production, urease production, growth rate at 37 °C, and hemolytic activity on Blood agar. One hundred milk samples were assembled from the White Gold village and surrounded outlying farms of Abu-Ghraib, Baghdad province, during the period from November 2018 to March 2019. Each milk sample was tested for California Mastitis (CMT). The results indicated that 85% of the samples gave positive (+ve) results for CMT. Sixty six mycotic isolates were detected, including 31 isolates of *Penicillium spp.* (46.9%) and 23 isolates of *P. chrysogenum* (34.8%). All of *P. chrysogenum* isolates were identified by culturing on Sabouraud Dextrose Agar and Czapek Doxes Agar at 25 °C for 5-7 days. *P. chrysogenum* was diagnosed by polymerase chain reaction (PCR) based on the internal transcribed spacer (ITS) region of fungal ribosomal DNA (rDNA). The results of genetic identities showed that this fungus had 94% matching with the reference strain. Also, this study indicated that *P. chrysogenum* has several virulence factors with the ability of this fungus to degrade both proteins (albumin and casein), hydrolyse urea, and grow at 37 °C, but not to confer hemolytic activity on Blood Agar.

**Keywords:** *Penicillium chrysogenum*, bovine mastitis, PCR, virulence factors, proteinase production, urease production.

## دراسة قوة ضراوة فطر *Penicillium chrysogenum* المعزول من حالات تحت السريرية لالتهاب الضرع في الابقار

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### الخلاصة

هدفت الدراسة الحالية الى العزل والتحري عن *Penicillium chrysogenum* من التهاب الضرع تحت السريري في الابقار. والى تقييم قوة اهم عوامل الضراوة بواسطة تقييم انتاج proteinase وانتاج urease و معدل النمو بدرجة 37 °C وفعالية التحلل على اكار الدم. جمعت 100 عزلة من الحليب من منطقة بغداد والحقول النائية المحيطة من منطقة ابو غريب خلال الفترة من تشرين الثاني 2018 الى شهر اذار 2019، وعملت كل عزلات الحليب بفحص CMT.

حيث شخصت 66 عذلة فطرية كانت 31 عذلة من *Penicillium spp.* بنسبة 46.9% و 23 عذلة من فطر *Penicillium chrysogenum* بنسبة 34.8% من العزلات الفطرية. تم التحري عن كل عزلات فطر *Penicillium chrysogenum* بواسطة زرعها على وسط Sabouraud Dextrose Agar و Czapek Doxes Agar بدرجة 25 °C لمدة 5-7 ايام. اضافة الى ذلك تم تشخيص *Penicillium chrysogenum* بواسطة فحص PCR بنجاح اعتمادا على منطقة ITS في DNA الفطري واطهرت نتائج التطابق الجيني لهذا الفطر بان نسبة التطابق وتشابه السلالة المرجعية 94% . كشفت هذه الدراسة ايضا بان فطر *Penicillium chrysogenum* يمتلك العديد من عوامل الظراوة مع قدرته على تحليل كلا بروتيني (الالبومين و الكازين) اضافة الى قابليته على تحليل اليوريا وقابليته على النمو بدرجة 37 °C، لكن لم يلاحظ فعالية التحلل على وسط اكار الدم.

## Introduction

Mastitis is one of the principal pathologies in the dairy bovine oppression. The majority of cases are caused by bacteria [1], but there are also cases caused by fungi [2]. Mycotic mastitis has become an increasing problem in animals and humans due to the wide use of antibiotics in mastitis therapy [3, 4]. A previous study [5] isolated several types of fungi from mammary secretions of lactating cows and buffaloes, including *Penicillium spp.*, especially *Penicillium chrysogenum*.

*Penicillium chrysogenum* is a filamentous fungus [6] which belongs to the family Trichocomaceae [7], genus *Penicillium*, and characterized by the typical brush-shaped morphology of its conidiophores [8]. The reproduction of this fungus is only asexual and due to the economic importance of this fungus which lead to revolution of sexual stage [9]. It is found in soil, wood, and decaying vegetation, and because of its dry, one-celled conidia, it is airborne [10]. Also, it is usually found in indoor environments and associated with food spoilage. It is known as an industrial producer of b-lactam antibiotics, particularly penicillin. The current production strains result from several decades of classical strain improvement (CSI) [11].

Polymerase chain reaction (PCR) is a rapid diagnostic technique that can be used for the detection of numerous fungal species with good pretest and posttest probabilities [12]. In fungi, PCR depends on the internal transcribed spacer (ITS) regions of fungal ribosomal DNA (r DNA) for the identification of many fungi, including *P. chrysogenum* [13]. This ITS region in fungi consists of 3 sub-regions, namely ITS1 and ITS2, which are considered as variable regions, and 5.8S gene, which is considered as the highly conserved region [14].

Virulence factors are products or a property of fungus that increases its ability to attack the host [15]. *P. chrysogenum* has the potential to produce many of virulence factors like proteases which are degradative enzymes [16, 17]. Other properties of this fungus include growth at 37 °C [18], hemolytic activity [19], and production of urease [15].

Very little is known about isolation and detection of virulence factors of *P. chrysogenum*. The objectives of this study are to give a special attention for the isolation and identification of *P. chrysogenum* besides studying the potential of virulence factors of this fungus isolated from subclinical bovine mastitis.

## Materials and methods

### Collection of milk samples

One hundred, four quarter, milk samples were collected from seemingly healthy cows from different areas in Baghdad region and the surrounded outlying farms of Abu-Ghraib during November 2018 to March 2019. Before sampling, the teat end was cleaned with 70% ethyl alcohol with dry and the first three steams of milk were discarded. Ten ml of milk were collected in sterile test tubes under aseptic condition, then immediately transported to the laboratory on ice [20].

### Detection of Subclinical Mastitis

All milk samples were subjected to CMT test by taking two ml of each sample, mixing it with an equal volume of CMT reagent (CMTR, Immucell, USA) in a plastic paddle, and shaking gently in a horizontal plane. The reactions were scored within 10-15 sec. of mixing and graded as +, ++, +++ for positive, trace and negative [20].

### Source of fungus isolates

*P. chrysogenum* was isolated from milk samples from cows infected by mastitis (subclinical) as tested by CMT. These isolates were cultured on Sabouraud dextrose Agar (Himedia -India) containing 0.05 mg/ml of chloramphenicol. The samples were then incubated at 25±2 °C for 4-7 days and diagnosed macroscopically and microscopically [21]. Urease production was also tested in samples cultured on Czapek Dox Agar (Oxoid -England).

### DNA extraction

The pure isolates of fungus, already grown on Sabouraud Dextrose Agar at 25 C for 7 days, were used for DNA isolation. Genomic DNA was extracted from fungal isolates according to the protocol of ZR Fungal/Yeast/Bacteria DNA mini prep Kit (Zymo/USA) following the instructions of the manufacturer. The concentration and purity of DNA were estimated by spectrophotometer at 302nm.

### Agarose gel electrophoresis of DNA

Electrophoresis was applied to determine DNA fragments[22].

### Preparation of samples

Three µl of the processor loading buffer (Intron / Korea) was mixed with 5 µl of the DNA supposed to be used for electrophoresis (loading dye). After the mixing process, the mixture was loaded into the holes of the gel. An electrical current of 7 v/c2 was applied for 1-2 h till the tincture reached to the other side of the gel. The gel was tested by a source of UV with 336 nm after putting the gel in a pool containing 3 µl Red safe Nucleic acid (Intron / Korea) staining solution and 500 ml distilled water. In addition, KAPA universal ladder kit was employed for determining the approximate size and quantity of double-stranded DNA on agarose gel.

### Primers design

Molecular identification was performed using the primers designed in this study and manufactured by Integrated DNA technologies/USA). The sequences of the fungal gene primers and the amplicon size are demonstrated in Table-1.

**Table 1-** The sequences of the fungal gene primers and the amplicon size.

Gene name	Primer	Sequence	Tm (°C)	GC (%)	Product size(base pair)
ITS	Forward	5'- TCCGTAGGTGAACCTGCGG -3'	60.3	50%	650
	Reverse	5' TCCTCCGCTTATTGATATGC- 3'	57.8	41%	

### Detection of gene ITS by using PCR

Detection of *ITS* gene was conducted by using primers for amplification. A fragment of *ITS* was amplified using a forward primer (*ITS1* F: 5'- TCCGTAGGTGAACCTGCGG -3') and a reverse primer (*ITS4* R:5' TCCTCCGCTTATTGATATGC-3') supplied by IDT (Integrated DNA Technologies company, Canada.). The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl Taq PCR PreMix (Intron, Korea), and 1µl of each primer (10 pmol). Then, distilled water was added into the tube to a total volume of 25µl. The thermal cycling conditions were conducted as follows: Denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 45s, 52°C for 1 min, and 72 °C for 1min, with a final incubation at 72 °C for 7 min using a thermal Cyclor (Gene Amp, PCR system 9700; Applied Biosystem). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red stain staining (Intron Korea ). In addition, the step of DNA gel extraction was performed according to a previously published procedure [23] by adding absolute ethanol to the wash buffer prior to initial use.

### Sequencing and Sequence Alignment

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violate light (302 nm) after Red Stain staining. Sequencing of gene was performed by the biotechnology lab, national instrumentation center for environmental management (NICEM), Korea,, using DNA sequencer 3730XL (Applied Biosystem). Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program, which is available at the National Center of Biotechnology Information (NCBI), and BioEdit program.

### Detection of virulence factors of *Penicillium chrysogenum*

**Albumin:** The hydrolysis of albumin was prepared by well diffusion method [24]. Two wells were made in a proteinase production medium which contained bovine serum albumin, prepared by taking 68 ml of a solution composed of MgSo<sub>4</sub>.7H<sub>2</sub>O( 0.04)g, K<sub>2</sub>Hpo<sub>4</sub>( 0.5)g, Nacl (1)g, yeast extract(0.2)g, glucose (4)g and bovine serum albumin (BSA) (0.5)g [25]. The pH of this medium was adjusted to 4, then the solution was sterilized by filtration using Millipore filter (0.22)µm. The medium was mixed with 140 ml of agar which was sterilized in autoclave at 121 °C under 15 pound/inch<sup>2</sup> for 15 min, then poured into sterile petri dishes using sterilized cork borer (5mm) with micropipette. Small parts of fungus ( 5mm) were then placed in these wells, and the petri dishes were incubated at 25 °C for 5-7 days. The proteolysis mold appeared as surrounded by a translucent clear zone due to the hydrolysis of albumin.

**Casein:** The hydrolysis of casein was prepared by well diffusion method [24]. Three wells were made in a skim milk agar medium composed of potato dextrose agar with 10% sterile skim milk added just before pouring the plates. This medium was prepared with modification of a previously reported method [26] using sterilized cork borer (5mm) with micropipette and cut out of the agar. Then a small part of this fungus was placed in the wells and the petri dishes were incubated at 25 °C for 5-7 days. The proteolysis mold appeared as surrounded by a translucent clear zone due to the hydrolysis of casein.

**Hemolytic activity:** *P. chrysogenum* was grown on SDA medium supplemented with 5% glucose and 5% sheep blood. Then, the petri dishes were incubated at 25 °C for up to 7 days and the hemolytic activity was classified as absent, partial, or total [27].

**Urease test:** *P. chrysogenum* was grown in a tube containing urea agar by stabbing and incubated at 25 °C for 3-5 days. The appearance of red or pink color in the medium indicated a positive result, evidencing raised PH (alkalinisation) due to urea breakdown and subsequent medium color change [15].

**Growth on 37°C:** The growth assay at 37°C was carried out in a petri dish containing SDA medium and *P. chrysogenum* was inoculated into 2 different petri dishes, one was incubated at 25 °C and the other at 37°C, in order to evaluate the ability of this species to develop in above-ambient temperature [15].

### Results and discussion

#### California mastitis test (CMT)

Out of a total of 100 samples of milk isolated from cows with mastitis (subclinical), which were examined from Abu-ghraib regions by California test, 85 (85%) samples showed positive (+ve) results for CMT and 15 (15%) samples confirmed negative (-ve) results (Table- 2). This result agree with that reported previously [28] and is caused by the interaction of host pathogens and environmental factors [29]. It can also be due to unhygienic conditions and prolonged treatment with the antibiotic, which may lead to increase the risk of fungal infection [30]. In addition, this study showed that sub clinical mastitis was more prevalent than clinical mastitis, which is similar to earlier results [31, 32].

**Table 2-**Results of California test

No. of milk samples	No. of (+ve) CMT samples	%	No. of (-ve) CMT samples	%
100	85	85%	15	15%

#### Percentage of mycotic mastitis

The results of 100 milk samples revealed that 66 (66%) had mycotic mastitis isolates, of which 31 isolates belonged to *Penicillium* spp. (46.9%), including 23 isolates of *P. chrysogenum* (23/66, 34.8%) , as shown in Table- 3. Several studies indicated the isolation of *penicillium* spp. from bovine mastitis [33, 34, 35]. One study [36] indicated that *Penicillium* spp., after *Candida* spp., was the predominant fungi in clinical and sub clinical mastitis. No previous study suggested the isolation of *P. chrysogenum* from bovine mastitis, due to very little attention to this topic and because this fungus was used to produce a type of antibiotics (penicillin) [37]. Mastitis remains an unsolved problem because of its

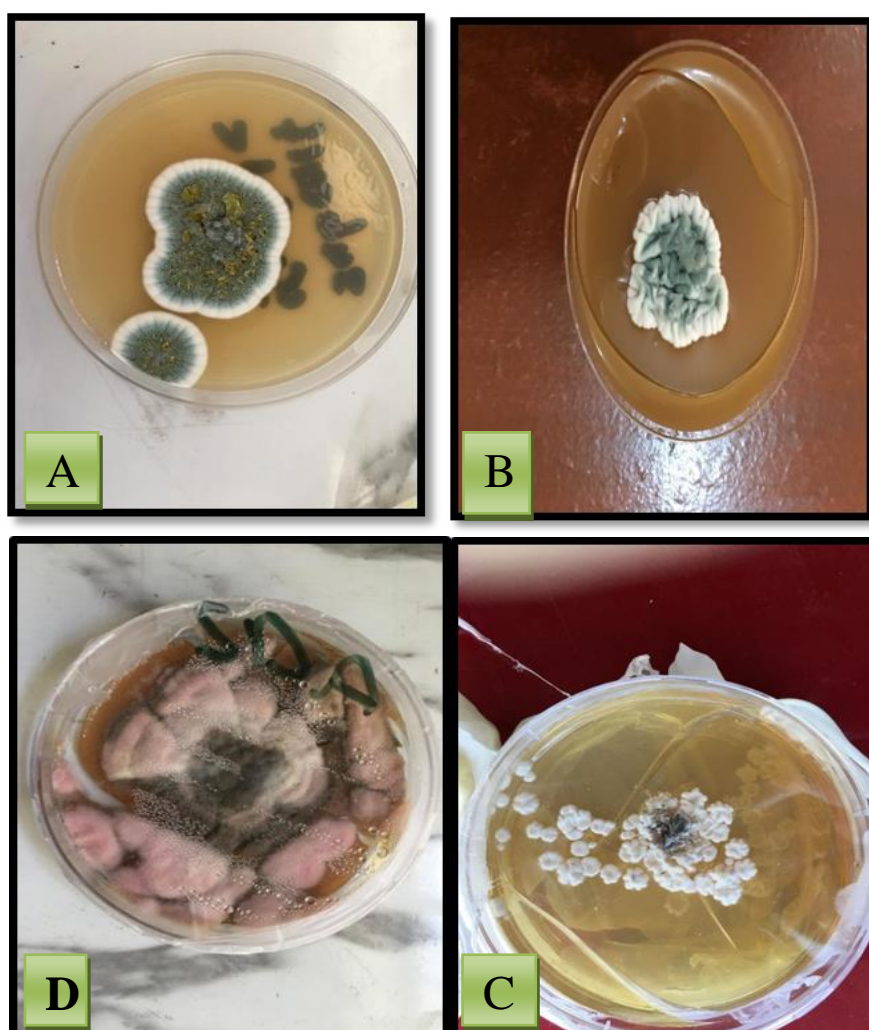
complex etiology [3]. Bovine mycotic mastitis was reported to be responsible for 1-13% of all mastitis cases [1]. The present study is considered the first to isolate this fungus from bovine mastitis in Iraq.

**Table 3-** Prevalence of mycotic infection in subclinical mastitis.....

No. of milk samples	No. of mycotic isolates	%	No. of <i>Penicillium</i> spp.	%	No. of <i>Penicillium chrysogenum</i>	% of <i>Penicillium chrysogenum</i> to mycotic isolates
100	66	66%	31	46.9%	23	34.8

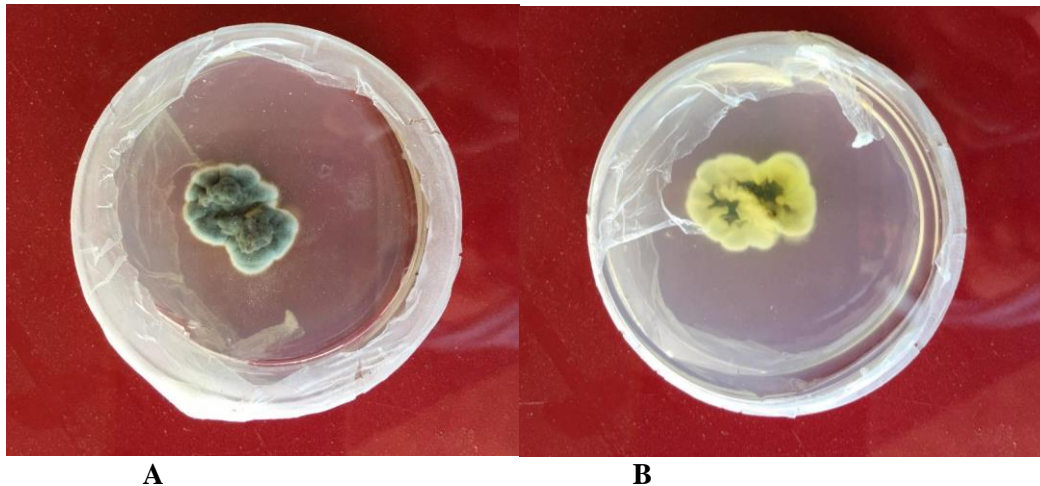
#### Identification of *Penicillium chrysogenum*

*Penicillium chrysogenum* was isolated and identified by subculture on SDA and incubation at 25°C for 5-7 days. The colonies appeared green or pale green-blue, changing to darker green with white margins, with velvety in texture. Exudate droplets were present in the center of colonies, having pale yellow to bright yellow color, as shown in Figure- (1-A), while this exudate was occasionally absent, as shown in Figure- (1-B). Also, the color of colonies sometimes appeared as white in color, as in Figure- (1-C), or changing to red or pink, as in Figure- (1-D). This is because many of *Penicillium* genera lose all of their green color with age, leading to various shades of yellowish brown and reddish brown to almost fuscous. The color of the colonies of the same species differs on different media [38].



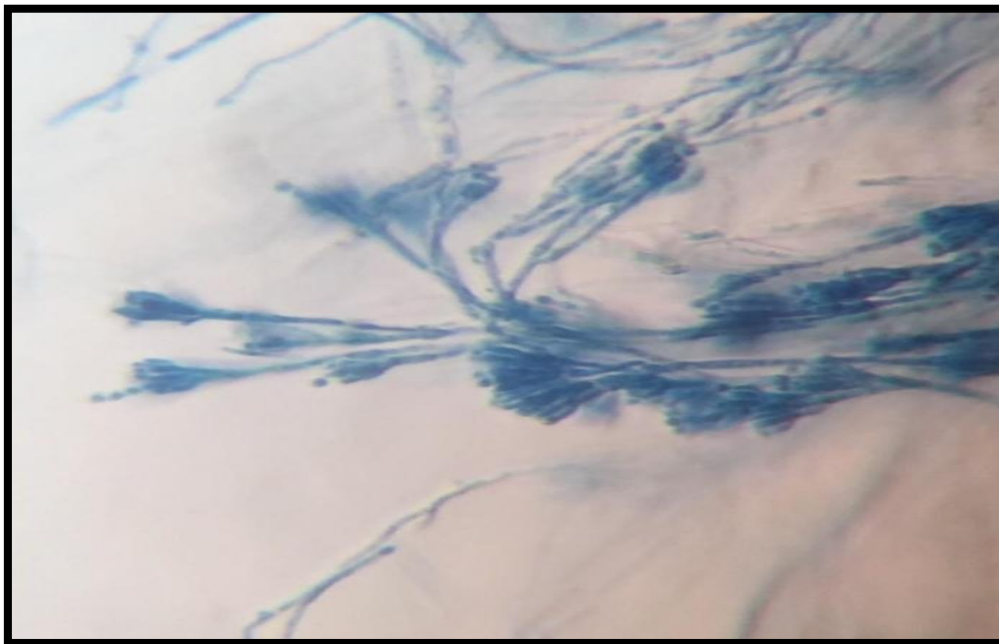
**A- Figure 1-** Macroscopically appearance of *P. chrysogenum* on SDA at 25 °C for 7 days; A- Exudate, B- Without exudate, C- White color colonies, and D- Red color colonies

The growth of this fungus was rapid on Czapek Doxes Agar and the color of colonies appeared pale green. The exudate was limited or absent, as in Figure- (2-A), with yellow pigment on the reverse side of the petri dish, as in Figure- (2-B).



**Figure 2-** Macroscopically appearance of *P. chrysogenum* on Czapek Doxes Agar (A), and the reverse side of Czapek Doxes Agar showing yellow pigment (B).

The microscopic examination of *P. chrysogenum* was performed by taking a small part of fungal growth and mixing it with one drop of lactophenol cotton blue stain. The specimen was covered with a cover slide and examined under microscope with 40x lens. This fungus appeared similar to the other *Penicillium* spp., showing a brush-like arrangement with septate hyaline hyphae. The conidia appeared subglobose to ellipsoidal with 2-3 stages of strigmata borne on the conidiophores, as shown in Figure-3.

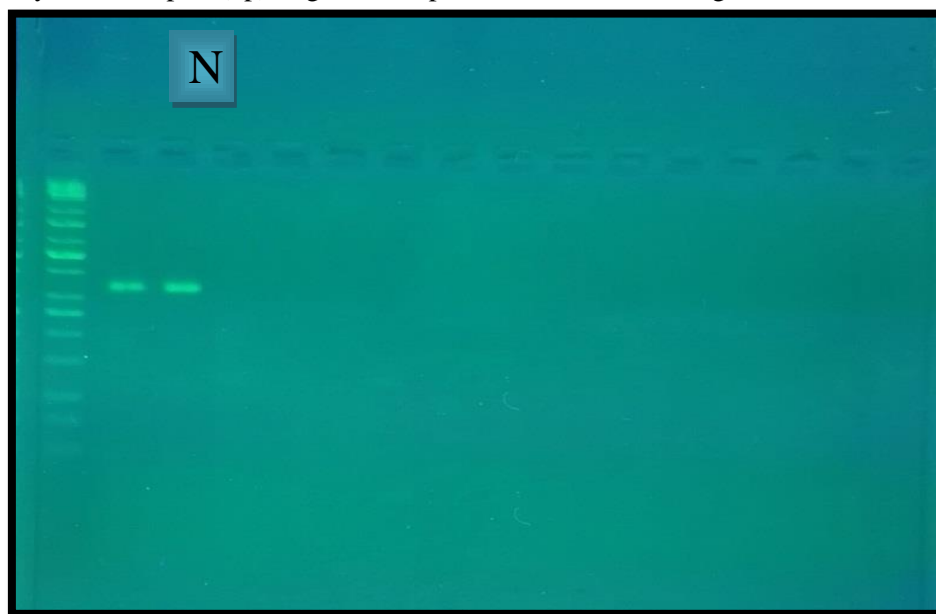


**Figure 3-**Microscopic appearance of *P. chrysogenum* by using lactophenol cotton blue stain (40X)

The macroscopic and microscopic features of *P. chrysogenum* were similar to the description provided by previous works [39, 40]. Also *P. chrysogenum* was different from *P. marneffeii* which is considered as a dimorphic fungus with the ability to especially infect humans and bamboo rats.

#### Molecular identification

*Penicillium chrysogenum* was identified based on the morphological characteristic as well as the genetic analysis of their ITS1, ITS2, 5.8S and 28S regions of rDNA. The results of the genetic identities showed that the isolate of *P. chrysogenum* had 94% matching with the reference strain. This strain of *P. chrysogenum* was found to be XQ23 with a sequences accession number of Ku216725.1. The amplification of ITS region was successful for this isolate and yielded a PCR band of approximately 650 base pair (bp) in gel electrophoresis, as shown in Figure- 4.



**Figure 4-** Gel electrophoresis on 2% agarose at 5 volt/cm<sup>2</sup> showing PCR product of *P. chrysogenum* at the band size 650bp. for 1:30 hours. N: DNA ladder

#### Virulence factors of *P. chrysogenum*

Virulence factors are properties which increase the survival, growth, and propagation of fungi in animal tissues [41]. From a total of 23 samples of *P. chrysogenum* isolated from 100 samples of milk, several virulence factors were evaluated and the results showed that all the isolates were able to degrade both proteins (albumin and casein) but in different degrees of virulence (higher, moderate and low). The inhibition zones of higher degrees of virulence for albumin and casein were 35 and 26 mm, respectively, shown in 4.34% and 13.04% of the isolates 1 and 3, respectively. Whereas, the inhibition zones of lower degrees of virulence for albumin and casein were 15 and 18.3 mm, respectively, shown in (13.04% and 8.69% of the isolates 3 and 2, respectively). The results are shown in Tables- 4 and 5 and Figures- 5 and 6, respectively. These results are in agreement with those of a previous study [15] which stated the ability of *P. chrysogenum* isolated from poultry barns to hydrolyse albumin and casein because this fungus contains hemolytic enzymes (proteinase enzymes). Another article [42] suggested that microbial cells secrete hydrolytic enzymes which destroy the constituents of host cell membranes, leading to membrane dysfunction and physical disruption, as well as the aid in the invasion of host tissues.

**Table 4-**The results of measurement of diameter of inhibition zone of albumin

Degree of virulence	No. of isolates	Percentage (%)	Diameter of inhibition zone (mm)
Higher	1	4.34	35
Moderate	3	13.04	20
Low	3	13.04	15
Very low	16	69.56	5-10

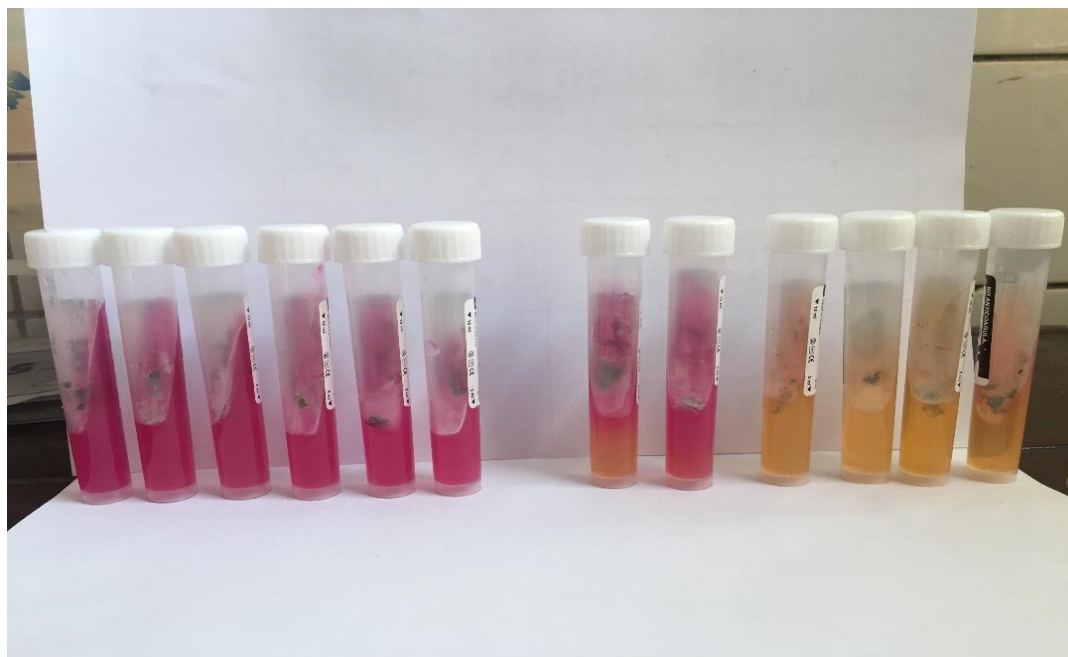
**Table 5-**The results of measurement of diameter of inhibition zone of casein

Degree of virulence	No. of isolates	Percentage (%)	Diameter of inhibition zone (mm)
Higher	3	13.04	26
Moderate	4	17.39	25
Low	2	8.69	18.3
Very low	14	60.86	10

**Figure 5-**Hydrolysis of albumin by *P. chrysogenum* proteinase production in the medium**Figure 6-**Hydrolysis of casein by *P. chrysogenum* on Skim Milk Agar

In addition, this fungus was able to hydrolyse urea and change the color from yellow-orange to pink. A total of 10 isolates (43.47%) of this fungus changed the color to dark pink, which indicates a higher degree of virulence. While, 5 isolates (21.73%) did not change color as shown in Figure-7 and Table-6. This result is in agreement with that of a previous study [15] which reported that *P. chrysogenum* was able to hydrolyse urea and has the capacity to hydrolyse urea, which is recognized as an important factor for pathogenicity of fungi.



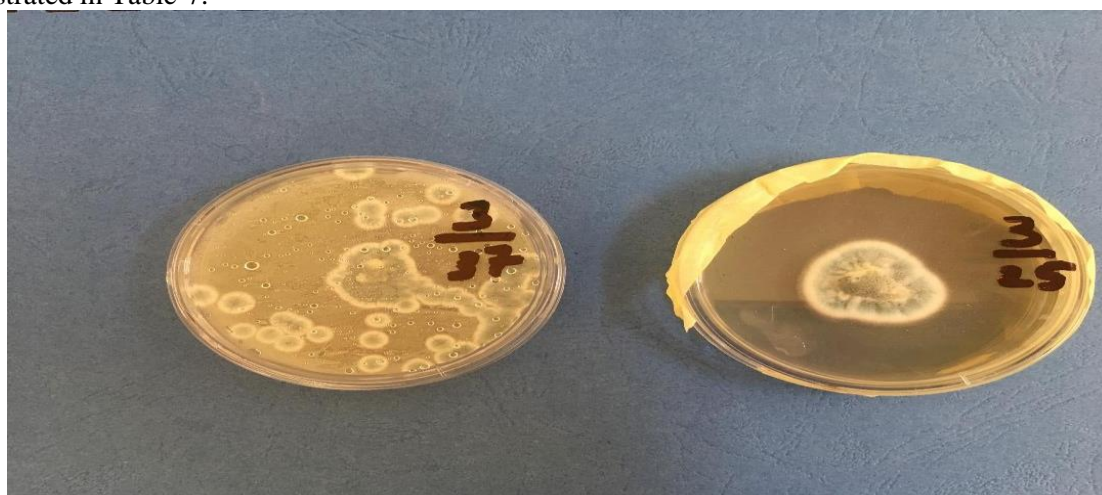


**Figure 7-** Hydrolysis of urea by *P. chrysogenum* on Urea Agar

**Table 6-**The results of growth of the isolates of *P. chrysogenum* on urea agar

Degree of virulence	No. of isolates	Percentage (%)	Color
Higher	10	43.47	Dark pink
Moderate	8	34.78	Light pink
No changing color	5	21.73	Orange-yellow

On the other hand, this fungus was able to grow on 25°C and 37°C, as shown in Figure-8. This result is highly similar to that of earlier works [15, 18, 43, 44] [44], which reported that *P. chrysogenum* can grow at 37°C despite their isolation from a very cold habitat, indicating that the fungus was thermo-tolerant. Thermo-tolerance is a crucial virulence factor for establishing the invasive type of infection in humans and animals, which is a trait that is lacking in most fungi. The other result of virulence factors of *P. chrysogenum* was the observation of no hemolytic activity on Blood Agar, as shown in Figure-9. This result is highly similar to that previously reported [15], but inconsistent with another study [45]. This inconsistency is possibly due to environmental conditions, source of isolate, and the method used in the detection of the hemolysin enzyme that may be produced from some *Penicillium* spp genera. However, another article [44] indicated that *P. chrysogenum* exhibited some degree of hemolysis on blood agar, only when incubated at 37°C, as well as all the virulence factors, as illustrated in Table-7.



**Figure 8-** *P. chrysogenum* growth on 25°C and 37°C for 7 days



**Figure 9-***P. chrysogenum* with no hemolytic activity on Blood Agar

**Table 7-** The results of virulence factors of *Penicillium chrysogenum*

Fungus	Albumin	Casein	Hemolysis	Urease	Grow on 37C
<i>Penicillium chrysogenum</i>	Positive	Positive	Absence of activity	Positive	Positive

### Conclusions

Mycotic mastitis, especially sub clinical mastitis, which occurs through fungal infection is a major problem due to the absence of signs of inflammation or milk abnormalities, causing economic losses and reduced milk production. This study highlighted that *P. chrysogenum* may cause bovine mastitis by the production of many types of virulence factors which are very important in pathogenicity of this fungus. This study is considered the first report about the isolation of *P. chrysogenum* from bovine mastitis in Iraq, revealing 34.8% cases of mycotic bovine mastitis. Hence, further studies are needed to investigate the other types of virulence factors and other types of *Penicillium spp.* which can be isolated from clinical and/or sub clinical isolates of bovine mastitis

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