The cytotoxicity effect of Metalloprotease produced and isolated from *Aeromonas* spp.

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Summary

One hundred and fifty stool sampling were collected from clinical sources responsible of causing diarrhea in adults as well as children. The identification of *Aeromonas hydrophila* isolates depended on common methods of identification dependent on biochemical characteristics and culture, then vitek2 compact system was used. Eight *Aeromonas hydrophila* isolates were gained and revealed various productivity of metalloprotease; the isolate number 8 was the maximum effective in metalloprotease production. The eight isolates were examined with Polymerase Chain Reaction to prove enzyme gene presence, the results revealed that all isolates were positive for *ahMP* genes; metalloprotease was completely purified via a number of steps, which included ammonium sulphate precipitation, dialysis, ion exchange and gel chromatography. Cytotoxicity effect of metalloprotease studies on cancer and normal cell lines, The results showed the purified metalloprotease as effective cytotoxic effect on liver hepatocellular cancer cells (HepG2) compared with no effect on normal liver cell line (WRL-68) indicating less cytotoxic effect.

Keywords: Polymerase Chain Reaction, Purification, Metalloprotease, Cell lines.

Introduction

Aeromonas species are well-known as causative of a varied range of diseases in animals and humans. Several studies were shown that a number of motile *Aeromonas* species were become food and marine pathogen of importance. They were been linked with numerous food-borne outbreaks and are gradually being isolated from patients who with traveler's diarrhea (1). The pathogenicity of *A. hydrophila* is multifunction. Surface associate factors content as example lipopolysaccharide, adhesins and the S-layer. All these factors include a collection of exotoxins and exoenzymes, for example enterotoxins, gelatinase, hemolysins, elastase, caseinase, lipases, lecithinase and proteases. Several of proteins cerebrated in pathogenicity are subject on the common secretory path for export (2). Currently, found that initial enteric pathogens, *Aeromonas* genus have the important characteristic for growing in aquatic systems, specifically in biofilms, where they may be resistant. *Aeromonas* species may be found in different numbers in water of drinking. The long period contact of immunocompromised persons to *Aeromonas* by polluted waters could possibly lead to aggressive disease, for example septicemia (3). Presently, several virulent elements have been studied and recognized in *A.hydrophila* include exotoxins, pili, adhesins and many exoenzymes for example proteases and amylases (4). The involvement of proteases to the virulence may be due to their capabilities to over above host lines and supply nutritional for the bacteria cells, so supporting invasiveness and establishing of disease (5). According to the above mentioned, this study performed aiming for to isolation and identification of *A. hydrophila* from patients suffering diarrhea and detection of metalloprotease gene in *A. hydrophila* isolates by Polymerase Chain Reaction technique, purification of metalloprotease produced by isolate and studying cytotoxicity effects of metalloprotease enzyme on normal and cancer cell lines.

Materials and Methods

A collection of 150 stool samples with aseptic technique were collected into sterilized cups from patients who were suffering of diarrhea and had certain clinical signs. Biochemical tests to identify the Gram-negative bacteria were done. Totally isolates of bacteria were identified via using VITEK 2

system. The capacity of *A. hydrophila* to metalloprotease producing was examined based on occurrence of halo zone round every colony (6). The recognition of metalloprotease presence gene via using polymerase chain reaction, the primer was designed in this study, by used blast program of NCBI (Table, 1) and this primer were used into amplification were specify for enzyme gene of *A. hydrophila*. The metalloprotease was completely purified according to (7) using ammonium sulphate precipitation, dialysis, ion exchange chromatography and gel chromatography filtration. The metalloprotease was studied for cytotoxic effect on cell line *in vitro*.

The cell line used was supplied by Al-Nahrain Center for Biotechnology, Al-Nahrain University. The 3- (dimethyl thiazol-2-yl)-2,5-biphenyl tetra zolium bromide (MTT) colorimetric assay was used to detect cell viability, MTT assay was achieved to determine effect of purified metalloprotease on two types of cell lines which were human liver hepatocellular cancer cells (HepG2) and normal embryonic liver cell line (WRL-68) for purified metalloprotease. Every cell-lines were cultured in flask and incubated with routinely condition (37ºC), the medium used for growing of cells was RPMI-1640. The suspension of cells was prepared to complete culture experimentations according to (8). The cell cytotoxicity effect of a number of concentration of metalloprotease enzyme (400, 200, 100, 50, 25, 12.5 and 6.25 µg/ml) on multiplying of the adherence cells in 96-well microtiter plate were accomplished according to (9). The cell viability was measured via using Optical Density (O.D.) of every well measured according to (10) as following:

$$\text{Cells viability \%} = \frac{\text{O.D. of test}}{\text{O.D. of control}} \times 100$$

Table, 1: PCR oligonucleotide primer.

Gene name	Primer sequence	Product size	Tm	Reference
ahMP	5'-CTGCAGAACA G G TGGATGTG-3' 5'GAGACCAGATA GACCAGGCG-3'	400 bp	64°C	In this study

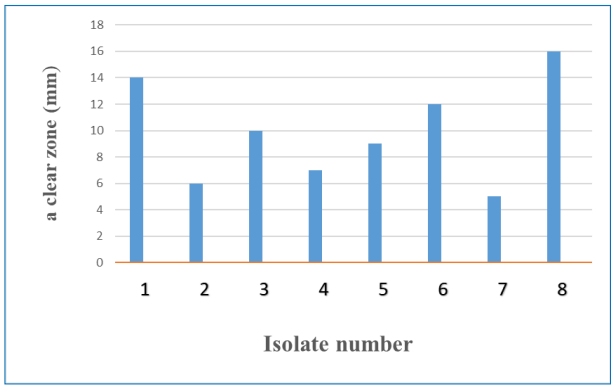
Results and Discussion

All isolated bacteria were examined by culture characteristics microscopally, Gram stain, Biochemical tests and Vitek 2 system, which is a phenotypic system used for identification microorganisms depending on biochemical reactions among the bacterial isolates suspended in an individual tubes of solution and media into the VITEK2 Identification Cards, to identify samples. 8 isolates (5%) were identified as *A. hydrophila*, while 142 (94%) samples which belonged to other bacteria were ignored, the current results of this study were in agreement with (11) who mentioned that the percentage of isolation of *A.hydrophila* from stool samples was 2.7%. Also in a previous study by (12), it was reported that (2.3%) of stool samples were *A. hydrophila*.

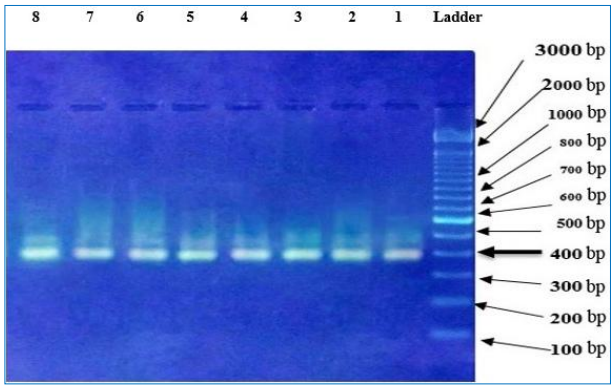
The results have shown that all isolates of *A. hydrophila* were able to enzyme production and have ability to hydrolysis of casein protein and formation clear zone around colonies. Results showed the eight isolates are able to form a halo a clear zone on enzyme production medium with different degrees, the halos diameters varied between 5 and 16mm depend on the isolate (Fig. 1), the highest production was shown by isolates code number (8) with 16mm in diameter of halo zone. The differences in the ability of the isolates to produce metalloprotease are due to genetic variations of the genes responsible for the production of metalloprotease and may be contributed to duration of sample collection, nutrition, source of samples, etc. (13).

The results of this study revealed that all bacterial isolated possess this gene, for this, we are a quite sure that the bacteria included in this study has ability to metalloprotease production. *A .hydrophila* showed the expected PCR amplification fragment of 400bp for *ahMP* gene, results of this study show that all isolates (100%) were positive in PCR (Fig. 2), the result of current study was similar to (14) who founded presence of gene in all isolates (100%) possessed *ahMP* gene, Similarly (15) who founded that (80%) of *A.hydrophila* isolated were positive to *ahMP* gene, but our results differed from previous study by (16) who reported that 53 from 193 isolates were positive for *ahMP* gene.

The obtained purification fold and recovery were 17.7 and 30.2% respectively. This result agrees with (17) who report the specific activity of metalloprotease extracted from *Aeromonas salmonicida* was enzymic yield was 39%, (18) indicated that specific activity of metalloprotease purified from *Aeromonas veronii* enzyme yield was 32%.



Figure, 1: Diameter of halo around colonies isolates on enzyme production medium.



Figure, 2: Agarose gel electrophoresis of PCR amplified products from extracted *Aeromonas hydrophila* DNA amplified with primer *ahMP*.

The cytotoxicity effects of metalloprotease by MTT assay was achieved to determine the effect of purified metalloprotease on two types of cell lines which were human liver hepatocellular cancer cells (HepG2) and normal embryonic liver cell line (WRL-68). Results show that the purified metalloprotease have cytotoxic effect on HepG2 cells line after 24 hours of incubation at 37 ºC (Fig. 3), while it was showed that less cytotoxic on WRL-68 cells (Fig. 4). Our result may explain how the tumor (cancer) cells has friable weak connective tissues proteins allowed degradable by metalloprotease, but the normal cell has strong extracellular matrix proteins and may need a long time or high concentration of enzyme to get degradation of cells. Similar results were recorded by (19), who found that the alkaline phosphatase enzyme produced by *Escherichia coli* had significant cytotoxic effect on cancer cell lines and slight cytotoxic effect on normal cell line while the study of (20) showed that the purified alkaline protease enzyme from *Vibrio* has cytotoxic effect on cell line.

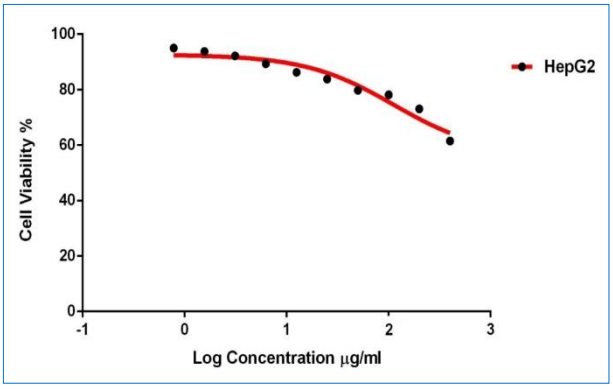
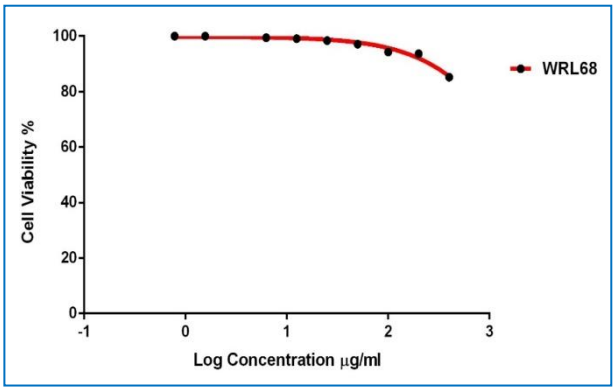


Figure , 3: The activity of purified enzyme on HepG2 cell line viability.



Figure, 4: The activity of purified enzyme on WRL-68 cell line viability.

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التأثير السمي لإنزيم البروتييز المعدني المنقى من بكتريا *Aeromonas hydrophila*

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الخلاصة

جمعت 150 عينة خروج من مرضى يعانون من إصابات الإسهال لعزل بكتريا *Aeromonas hydrophila*، حُصّلت من هذة العينات على 8 عزلة بكتيرية إخضعت جميعها إلى التشخيص على أساس صفاتها المظهرية والمزرعية وخصائصها الكيموحيوية وقد أشارت نتائج التشخيص إلى أن هناك 8 عزلة بكتيرية من بين العدد الكلي للعزلات. أجري فحص تفاعل إنزيم البلمرة التسلسلي للتحري عن نسبة وجود الجين البروتييز-المعدني الذي هو مسؤول عن إنتاج إنزيم البروتييز-المعدني بوساطة تقنية تفاعل البلمرة التسلسلي وكانت جميع العزلات حاوية على الجين، غُربِلتْ العزلات البكتيرية على أساس قابليتها على تحلل بروتين الكازائين وتكوين هالة شفافة حول المستعمرات. أشارت النتائج إلى أنّ جميع هذة العزلات كانت منتجة للبروتيز ولكن بدرجات متفاوتة، وقد تميزت من بينهما العزلة البكتيرية رقم 8 في انتاجيتها العالية للبروتيز-المعدني، لذا فقد انتُقِيَتْ هذه العزلة لقابليتها في إنتاج الإنزيم. نُقِيَ الانزيم المنتج تحت الظروف المثلى بأربع خطوات تضمنت الأولى الترسيب بكبريتات الامونيوم، الديلزة، التبادل الايوني والترشيح. دُرِسَ تأثير السمية الخلوية لإنزيم البروتييز-المعدني للكشف عن التأثيرالسمي الخلوي للانزيم على الخلايا الطبيعية والسرطانية باستعمال طريقة MTT. اذ اظهرت النتائج التأثير السام لإنزيم البروتييز-المعدني على خلايا (HepG2) باستعمال تركيز قليل من الانزيم، ومقارنة مع خلايا (WRL-68) اذ لم يكن له تأثيرا يذكر.

الكلمات المفتاحية: تفاعل بلمرة تسلسلي، تنقية، بروتييز معدني، خطوط خلوية.