A BACTERIOLOGICAL STUDY ON *ARCANOBACTERIUM HAEMOLYTICUM* ISOLATED FROM HUMAN PHARYNGITIS IN AL-HILLA CITY

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillments of the Requirements for the Degree of Master in Medical Microbiology

By

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٢٠٠٦ A.D **١٤٢٧ A.H**

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا

إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ

صَدَقَ اللهُ الْعَظِيمُ

سورة البقرة: الآية (٣٢)

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Dedication

To . . .

My Family

Acknowledgements

I am deeply indebted to my supervisor, Assistant Professar Mohammed Sabri Abdul-Razzak, Ph.D., Head of Microbiology Department, College of Medicine, University of Babylon for his guidance, patience, and supervision. This thesis would not have been possible, without his sincere help and assistance.

My thanks are also due to all members of the Department of Microbiology for their generous co-operation.

Rusul

A BACTERIOLOGICAL STUDY ON *ARCANOBACTERIUM HAEMOLYTICUM* ISOLATED FROM HUMAN PHARYNGITIS IN AL-HILLA CITY

ABSTRACT

A Total ٣٠٠ throat swabs were obtained from patients of both sexes suffering from acute or chronic pharyngitis (children and adults) and admitted to Hilla Teaches Surgical Hospital . It was found that only eight isolates of *A.haemolyticum* were identified. Three of them were isolated from female and five from male patients whereas no isolates of *A.haemolyticum* were found in the healthy controls.

Colony morphology and type of hemolysis on blood agar divided *A.haemolyticum* isolates into smooth and rough colony type. In this study, smooth and rough colony types were seen on human blood agar after ٤٨hrs. of incubation. It was observed that three isolates were of the smooth type and five of the rough one.

Some virulence factors of bacteria were also studied, the results showed that all bacterial strains produced lipase and phospholipase D but not protease. The ability of the bacterial isolates to produce hemolysin was also investigated and it was found that only three isolates were able to produce hemolysin and these isolates were smooth type, whereas five isolates had no ability to produce hemolysin and they were of rough type. The results also showed that the strains were positive for reverse CAMP test which had capacity to block the hemolytic activity of the beta- lysine (phospholipase C) produced by *Staphylococcus aureus.*

The effect of temperature on the bacterial growth was also studied. It was found that *A.haemolyticum* could grow well at ٢٥, ٣٠, ٣٥, ٤٠^{o}C as well as ٤^{o}C. This bacterial growth was inhibited completely at a temperature above ٤٠^{o}C. The same profile of temperture was seen on both smooth and rough isolates of *Arcanobacterium haemolyticum* under investigation.

The effect of some antibiotics on *A.haemolyticum* was investigated, the results showed that all isolates were sensitive (١٠٠٪) to erythromycin, penicillin and azithromycin. On the contrary, all isolates were resistant (١٠٠٪) to gentamycin whereas some isolates showed resistance in lesser degrees to vancomycin (٦٢.٥٪) to cefotaxime (٥٠٪), and to cephalexin, clindamycin and ampicillin

(٣٧٪) . The isolates were also resistant to deoxycycline, ciproflaxine and tetracycline at a rate (٢٥٪).

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LIST OF ABBREVIATIONS

PLD	Phospholipase D
CAMP	Christie, Atkins, and Munch-Peterson
PC	Phosphatidylcholine
PA	Phosphatidic Acid
LPA	Lipid mediator Lysophosphatidic Acid
TAG	Triacyllycerol
DNase	Deoxyribonuclease
TSA	Tryptic Soy Agar
TCA	Trichloroacetic Acid

دراسة بكتريولوجية على

Arcanobacterium haemolyticum

المعزولة من حالات التهاب البلعوم في مدينة الحلة

الخلاصة

من بين مجموع ٣٠٠ مسحة بلعمية تم جمعها من المركز الاستشاري لمستشفى الحلة الجراحي استحصلت من المرضى الذين يعانون من التهاب البلعوم (الحاد أو المزمن) ومن كلا الجنسين, تم عزل وتشخيص ثمان عزلات من بكتريا *Arcanobacterium haemolyticum* : ثلاث عزلت من الإناث وخمس من الذكور . في حين لم يتم عزل البكتريا من الأشخاص غير المصابين .

أظهرت النتائج اعتماداً على قابلية البكتريا على إنتاج إنزيم الهيمولايسين والصفات المظهرية للمستعمرة على تقسيم البكتريا إلى النوع الخشن والنوع الناعم على أكار الدم المغذي ، إذ لوحظ وجود ثلاث عزلات من النوع الناعم وخمس عزلات من النوع الخشن .

درست بعض عوامل الضراوة لهذه البكتريا ، وقد وجد أن جميع العزلات موجبة لإنتاج إنزيم اللايبيز والفوسفولايبيز D ، في حين لم تظهر العزلات قابلية على إنتاج البروتيز .

كما درست قابلية البكتريا على إنتاج إنزيم الهيمولايسين من النوع β ، إذ وجدت ثلاث عزلات فقط لها القابلية على إنتاج الهيمولايسين حيث إن هذه العزلات من النوع الناعم ، في حين لم تظهر العزلات الباقية قابلية لإنتاج الهيمولايسين والتي تعود إلى النوع الخشن .

وقد أظهرت النتائج أن جميع العزلات موجبة لفحصCAMP العكسي حيث وجد لها القابلية على تثبيط الفعالية التحليلية لإنزيم β-lysin المنتج من قبل بكتريا *Staphylococcus aureus* .

وتم أيضاً دراسة تأثير الحرارة على نمو البكتريا ، وقد لوحظ بأن البكتريا تنمو عند درجات الحرارة (٤ – ٤٠)°م ، كما أن الحرارة فوق(٤٠ °م) تثبط النمو البكتيري بشكل كامل. وقد لوحظ أيضاً إن التأثير الحراري كان مماثلا على العزلات الناعمة والخشنة من بكتريا *Arcanobacterium haemolyticum* .

درس تأثير بعض المضادات الحياتية على عزلات بكتريا *A.haemolyticum* وأظهرت النتائج بان جميع العزلات حساسة لكل من الارثرومايسين والبنسلين والازوثرومايسين في حين على العكس لوحظ بان جميع العزلات مقاومة وبشكل كامل للجنتامايسين وبدرجة اقل من الفانكومايسين (٦٢.٥%) إضافة إلى الكلنداميسين والامبسلين (٣٧%) ، وأيضاً للدوكسيسايكلين وسبروفلاكسين والتتراسايكلين (٢٥%) .

دراسة بكتريولوجية على

Arcanobacterium haemolyticum

المعزولة من حالات التهاب البلعوم في مدينة الحلة

رسالة

مقدمة إلى مجلس كلية الطب في جامعة بابل كجزء من متطلبات نيل

درجة الماجستير في علم الأحياء المجهرية الطبية

من قبل

رسل عدي هاشم

بكالوريوس علوم (الأحياء المجهرية)

١٤٢٧ هـ **٢٠٠٦ م**

Chapter One

Introduction and Literature Review

١.١ Introductions

Arcanobacterium haemolyticum (formerly *Corynobacterium haemolyticum*) is an aerobic gram positive rod first found in ١٩٤٦ in persons with pharyngitis and skin infections (Maclean, ١٩٤٦).

A. haemolyticum has been implicated as an important cause of pharyngitis in adolescents and young adults, frequently causing an exanthema that may mimic viral exanthema, toxic erythema or drug eruption which usually resolve in few days with or without therapy (Gaston, ١٩٩٦).

Serious infections such as brain abscess, meningitis, septicemia, endocarditic and osteomyelitis occur less frequently but are fatal if not treated early (Hoosen, *et.al.* ١٩٩٠); other less serious infections include chronic skin ulcers, cellulites and otitis media (Bhat, *et.al.*, ١٩٩٧).

The taxonomical position of *A. haemolyticum* has caused confusion in the past, largely because of its close phenotypical similarity to *Actinomyces pyogenes* (formerly *Corynebacterium*

pyogenes), which is primarily an animal pathogen (Coyle and Lipsky, ١٩٩٠).

Antibiotic susceptibility tested on human blood agar have showen that *A.haemolyticum* is susceptible to many types of antibiotics however, some strains show resistance to certain antibiotics (Biswas, *et.al.*, ٢٠٠٣) .

Infections is most common in ١٥-٢٥ years old persons, and is thought to result from droplet transfer from infected persons (Gaston and Zurowski, ١٩٩٦).

The demonstration that *A. haemolyticum* is not a component of the human commensal flora is essential to establishing its role in human infection (Mackenzie, *et. al*., ١٩٩٥).

There is no independent study on this bacterium conducted in Iraq, so this study is aimed to:

١. Isolation and characterization the bacteria associated with pharyngitis from patients (children and adults) from both sexes.
٢. Study the effect of some antibiotics on *A.haemolyticum* isolates.
٣. Study some factors associated with the pathogenecity of *A.haemolyticum* such as lipase, protease, and phospholipase D.
٤. Investigate the effect of temperature on the activity and survival of this bacterium.

١.٢ Literature Reviews

١.٢.١ General Features of *A.haemolyticum*

A.haemolyticum is a pleomorphic, gram positive rod, facultatively anaerobic, non motile and non sporulating. It can sometimes appear granular or beaded and resemble irregular cocci. The growth is enhanced by the addition of ٥-١٠٪ CO_2 (Clarridge, ١٩٨٩).

Optimal growth is obtained utilizing blood or serum enriched media incubated at ٣٧C^{o}. The colony size and degree of hemolysis vary with the type of blood cell (Cummings, *et.al*., ١٩٩٣).

Colonies on human blood agar after ٢٤ hour incubation are of ٠.٥mm diameter, and exhibit a narrow zone of hemolysis; at ٤٨ hours, the colony diameter is ١mm with hemolysis zone of ٣.٥mm (Cummings, *et.al.,*١٩٩٣).

On sheep or human blood agar, overnight colonies are tiny and little or no hemolysis is observed; at ٤٨-٧٢ hours, the haemolysis zone is about ١mm in diameter. Colonies are circular, discoid, opaque and whitish. A uniform feature of *A.haemolyticum* colonies is a black opaque dot at the center of colony which remains if the colony is scarped aside (Waagner, ١٩٩١). The colonies of *A.haemolyticum* have been suspected on the basis of the growing

time interval (٤٨), reduced size (diameter less than ١mm), the presence of a narrow zone of incomplete beta- haemolysis and the typical pitting of the culture medium beneath the colony. The definitive identification is realized on the basis of the microscopical appearance (diphtheroid gram- positive bacilli), negative catalase test and inverse CAMP test (Coman, *et.al.*, ١٩٩٦). *A.haemolyticum* does not produce catalase or indole. The bacterium does not hydrolyze esculine, gelatin and urea. Most strains do not reduce nitrate. *A.haemolyticum* produce acid from glucose, lactose and maltose, gives no hemolysis in CAMP test (reverse CAMP positive) and produces lipase, extracellular deoxyribonuclease (DNase) and phospholipase D (Krech and Hollis, ١٩٩١).

The peptidoglycan of *A. haemolyticum* contains lysine as the dibasic amino acid, glutamic acid and alanine are detected in the hydrolysates (Collins, *et.al.*, ١٩٨٢).

Colony morphology ,beta- hemolysis on sheep or human blood agar and the ability to ferment sucrose or trehalose defined two biotypes of *A.haemolyticum* : one which is the smooth type, grow as smooth, beta-hemolytic colony and fermented sucrose, whereas the other, the rough type ,grows as a rough colony and is non hemolytic and negative for sucrose and trehalose fermentation (Carlson, *et.al.*, ١٩٩٤). Both biotypes have extracellular amorphous polymeric

materials containing at least proteins. None of the strains posses a paracrystaline S (surface) layer (Lounatmaa, *et.al*., ١٩٩٣).

The smooth type is predominant in wound infections, whereas the rough type is isolated almost exclusively from respiratory tract specimens (Fell, *et.al.*, ١٩٧٧).

A.haemolyticum is accepted as an occasional human pathogen causing pharyngitis, some times associated with cervical adenitis and often with ascarlatiniform rash. *A.haemolyticum* has also been isolated from chronic skin ulcers and occasionally from other lesions such as brain abscess and osteomyelitis (Collee, *et.al.*, ١٩٩٦).

A.haemolyticum closely resembles *Actinomyces pyogenes* that is also gram-positive, catalase negative and produces beta-hemolytic colonies, however, the two organisms are easily differentiated as *Actinomyces pyogenes* hydrolyses gelatine rapidely, and produce acid from xylose (Goyal, *et.al.*,٢٠٠٥).

١.٢.٢ Taxnomy of *A. haemolyticum*

Initially named *Corynebacterium haemolyticum*, the bacterium now termed as *Arcanobacterium haemolyticum* ,was first described in ١٩٤٠ at the pathogenic agent causing pharyngitis and cutenous infections among US servicemen (Maclean, ١٩٤٦). As a result of its close resemblance to *Corynebacterium pyogenes,* some investigators

have believed the bacterium to be mutant of this species and appended a subspecies name, *Corynebacterium pyogenes* sub sp. homins (Collins, *et.al.*, ١٩٨٢).

The factors that have contributed to the earlier confusion surrounding the taxonomy of *A. haemolyticum* are the following:

١. Designation of the organism as *corynebacterium pyogenes* var. hominis by some investigators (Soucek and Souckov, ١٩٦٤).
٢. A report that it is mutant of *Corynebacterium pyogenes* (Barksdale, *et.al.,* ١٩٥٧).
٣. The description of positive catalase test that is not observed by conventional methods (Barksdale, *et.al.*, ١٩٥٧).
٤. The lack of hemolysis after ٢٤ hour incubation on sheep or human blood agar.

On the basis of biochemical tests and peptidoglycan, cell wall fatty acid and DNA data, *A.haemolyticum* have been removed from the genus *Corynebacterium.* It has been assigned to a new genus, *Arcanobacterium* (Latin “arcanus” = secretive), composed of this single species (Collins, *et.al.*, ١٩٨٢).

١.٢.٣ Pathophysiology of *A. haemolyticum*

A. haemolyticum has been implicated as an important cause of pharyngitis in adolescents and young adults, frequently causing an

exanthema that may mimic a viral exanthema, toxic erythema or drug eruption which usually resolves in a few days with or without therapy (Gaston and Zurowski, ١٩٩٦).

A.haemolyticum is an obligate pathogen, rarely isolated outside of the disease process. This organism causes pharyngitis and skin infections in human, although it can invade from the initial site of infection to cause meningitis, septic arthritis and endocarditis (Jost, *et.al.*, ٢٠٠١).

A.haemolyticum may cause peritonsillar and pharyngeal abscesses (White and Foshee, ٢٠٠٠) subperiosteal abscess, sinusitis (Ford, *et.al.*, ١٩٩٥), empyema with mycoplasma peumoniae infections (Stacey and Bradlow, ١٩٩٩), respirtary tract infections (Katkar, *et.al*., ٢٠٠٥), central nervous system infections, paravertebral abscess as a complication of ingrown toenail (Dieleman, *et.al.*, ١٩٨٩).Severe systemic infections such as endocarditis and meningitis have also been reported in immunocompromised patients (Minarik, *et.al.*, ١٩٩٧). Recently, Biswas, *et.al.,* (٢٠٠٣) have reported acase of chronic osteomyelitis due to *A.haemolyticum.* Pyothorax in an immunocompetent adolescent patient have been reported (Parija, *et.al.,* ٢٠٠٥).

The first case of septic arthritis caused by *A.haemolyticum* has also been reported. The wareness of the pathogenic potential of this

organism has been increases in bone and joint infections (Goyal, *et.al*., ٢٠٠٥).

A.haemolyticum has been described as an unusual pathogen causing pharyngotonsillitis and extrapharyngeal infections like ulcerative lesion infections mainly in patients with underlying conditions (peripheral vascular disease, diabetes, alcoholism) Panzara and Taranu, (٢٠٠١). There are increasing reports of systemic infections caused by *A.haemolyticum* (Parija, *et.al.*, ٢٠٠٥). Rarely is a patient with pharyngitis caused by *A.haemolyticum* hospitalized because of an inability to swallow. No deaths have been reported resulting from pharyngitis caused by *A.haemolyticum*, although *A.haemolyticum* have caused the death of two patients with endocarditis Chandrasekar and Molinari,(١٩٨٧).

A.haemolyticum also appears to be isolated from both immunocompetent and immunocompromised hosts (Alos*, et.al.*, ١٩٩٥).

A. haemolyticum has rarely been reported in animals and its pathogencity in animals has not been well documented (Robert, ١٩٦٩). This organism has not been identified as a part of the normal microflora of animals (Waagner, ١٩٩١). The role of *A.haemolyticum* as a zoonotic disease and its differentiation from the closely related animal pathogen *A.pyogenes* need to be defined (Billington, *et.al*., ٢٠٠٢).

Humans are believed to be its main environmental reservoir (Alonso, *et.al.,* ٢٠٠٢) although, usually, *A .haemolyticum* is not a respiratory colonizer.

A. haemolyticum has been isolated from pus drained from ankle joint, probably due to the spread of infections from abrasion over medial malleolus (Ford, *et.al.*, ١٩٩٥). Skin rash and predominant growth on multiple blood cultures suggest a causative role for *Arcanobacterium haemolyticum*.

Throat infections are often accompanied by cervical lymphadenopathy (Mackenzie, *et.al.,* ١٩٩٥). An erythematous morbilliform or scarlatinal rash of the trunk, neck, or extremities is associated with ٢٠٪ to ٢٥٪ of cases (Banck and Nyman, ١٩٨٦).

The mechanism responsible for adherence of *A. haemolyticum* to the pharyngeal mucosa is unknown (Karpathios, *et.al.,* ١٩٩٢). The pathophysiology of the rash is also unknown, However, the hypothesis that the rash is caused by bacterial exotoxin is reasonable.

Although initial attempts to identify toxins unproductive, phospholipase D (PLD), neuraminidase and hemolysin have been subsequently identified as extracellular toxin secreted by *A. haemolyticum* (Soucek and Souckov, ١٩٧٤).

Phospholipase D is known to bring about tissue damage, as elaborated by this organism as well as by the closely related bacterium, *Corynebacterium pseudotuberculosis,* an important pathogen of sheep (Linder, ١٩٩٧). Phosphlipase D shares many properties with Corynebacterial PLDs, including biochemical and biological activities is responsible for the toxicity of the venom of the brown recluse spider (Bernheimer, *et.al.*, ١٩٨٥).

١.٢.٤ Epidemiology of *A.haemolyticum*

A.haemolyticum infections is often reported from deliberate screening for the organism of a large number of patients with sore throat. Most cases involve pharyngitis or tonsillitis and approximately ٥٠٪ are exudative (Miller, *et.al*., ١٩٨٦).

A.haemolyticum occurs only after recurrent infections, which are thought to be related to incorrect initial diagnosis, resulting in less-than –optimum treatment (Banck and Nyman,١٩٨٦).

Infections is most common in ١٥-٢٥ years old persons, and is thought to result from droplet transfer from infected persons (Gaston and Zurowski, ١٩٩٦). Symptoms resemble those of beta- hemolytic Streptococci or viral infections. The spectrum of disease ranges from sore throat to, in rare cases, a life- threating membranous

pharyngitis resembling diphtheria (Green and Lapeter, ١٩٨١).

The demonstration that *A.haemolyticum* is not a component of the human commensal flora is essential in establishing its role in human infection (Mackenzie, *et.al.*, ١٩٩٥). Epidemiologic contact studies indicate that the infection likely spreads through an unknown route by human contact with people who are infected (Miller, *et.al.*, ١٩٨٦).

A.haemolyticum has rarely been recovered in healthy individuals (٠.٠٢٪), indicating the probable absence of a chronic carrier state, although direct inoculation studies indicate that transient asymptomatic colonization by *A.haemolyticum* can occur (Karpathios, *et.al.*, ١٩٩٢). *A.haemolyticum* most frequently causes opportunistic infection in immunocompromised patients.

A.haemolyticum occurs relatively often in polymicrobic infections together with typical respiratory pathogens such as *Streptococci* (Linder, ١٩٩٧). Is not yet determined if the presence of other microorganism contributes a synergistic role in the pathogenicity of *A.haemolyticum* (Skov, *et.al.*, ١٩٩٨).

Less frequent infection including osteomyelitis, meningitis and brain abscess can occur due to this bacteria (Funke, *et.al.*, ١٩٩٧).

A.haemolyticum has rarely been reported in animals and its pathogenicity in animals has not been well documented (Richardson

and Smithpj, ١٩٦٨). It is important to acknowledge the fact that this organism's presence is increases because some of the infections may become serious (Mebta, ٢٠٠٣).

Some ٠.٥٪ to ٣٪ of cases of pharyngitis can be traced of *A.haemolyticum* depending on the population studied with the highest number among ١٥-٣٠ years–old–patients. (Banck and Nyman, ١٩٨٦).

١.٢.٥ Virulence factors of *A.haemolyticum*

A.haemolyticum is known to produce un characterized hemolytic agents (Gaston and Zurowski, ١٩٩٦) and two biochemically defined extracellular products; a neuraminidase and phosphlipase D acting preferentially on sphingomyelin and generating ceramide phosphate in the target membrane (Souck, *et.al.*, ١٩٧١).

١.٢.٥.١ <u>Phospholipase D (PLD)</u>

Phospholipase D activity was first discovered in carrot extracts as a phospholipids- specific phosphodiesterase activity that hydrolyzed phosphatidylcholine to yield phosphatidic acid (PA) and choline (Hanahan and Chaikoff ١٩٤٧) .

Over ٥٠ years of biochemical studies culminating in the identification of PLD genes in the mid ١٩٩٠ have implicated PLD activity and its product PA in wide range of physiological processes and disease , including inflammation, diabetes (Hammond, *et.al.*, ١٩٩٥), cytoskeletal rearrangement (Cross, *et.al.*, ١٩٩٦), vesicle trafficking and exocytosis (Siddhanta, *et.al.* ٢٠٠٠), phagocytosis (Kusner, *et.al.*,١٩٩٦), neuronal and cardiac stimulation (Park, *et.al.*, ٢٠٠٠) , matrix metalloproteinase production (Reich, *et.al.*, ١٩٩٥), oncogenesis, (Welsh, *et.al.*, ١٩٩٤) , and the oxidative respiratory burst in neutrophils (Waite, *et.al.*, ١٩٩٨). In addition, exogenously added phosphatidic acid (PA) and PLD stimulate DNA synthesis in cells, indicating a potential role for PLD in mitogenesis (Exton, ١٩٩٤).

PLD activity appears to be present in most cell types (Meier, *et.al.*, ١٩٩٩), with the reported suggested exceptions of peripheral leukocytes and some lymphocyte lines (Bradshow, *et.al.*, ١٩٩٦).

PLD activities have been reported within the plasma membrane, cytosol, endoplasmic reticulum, Golgi and nuclei (Provost, *et.al.*, ١٩٩٦). The PLD gene from *A.haemolyticum* has been cloned and shown to have a high degree of homology with that of *C.pseudotuberculosis,* which it is thought to participate in vascular permeability and dissemination of the pathogen. (Cuevas and Songer, ١٩٩٣).

The activation of PLD in response to variety of hormones, growth factor and neurotransmitters catalyzes the hydrolysis of phosphatidylcholine (PC), generating choline and phosphatidic acid (Billah and Anthes ,١٩٩٠). Phosphatidic acid generally recognized as the signaling product of PLD functions as an effector in multiple physiological processes (Kim, *et.al.*, ٢٠٠٣).

PLD activity is present in a wide variety of cell types including blood platelets, fibroblasts, muscle cells and others (Kanfer, *et.al.,* ١٩٩٦).

PLD activity has been implicated in the regulation of cell growth (Ramires de Molina, *et.al.*, ٢٠٠١), membrane traffic and action dynamics (Cockcroft, ٢٠٠١), metastasis (Paii, *et.al.*, ١٩٩٤), the burst of neutrofiles (Suchard, *et.al.*,١٩٩٤) and specific signaling pathways (Liscovitch and Amsterdam, ١٩٨٩).

PLD enzymes is local inflammation by catalyzing the production of the lipid mediator lysophosphatidic acid (LPA) (Van Dijk, *et.al.*, ١٩٩٨).

PLD is known to bring about tissue damage as elaborated by *A.haemolyticum* as well as the closely related bacterium, *C.psudotuberculosis* an important pathogen of sheep (Linder, ١٩٩٧).

The enzyme was responsible for the dermonecrrotic, as well as the synergistic hemolytic activity of the organism that elaborate it (Soucek, *et.al.*, ١٩٧١). The role of potentiated cytotoxicity caused by the combined activity of PLD and cooperative agents such as

cholesterol data involving cophagocytosis (Linder and Bernheimer, ١٩٨٢).

PLD has been found to be markedly elevated in various cancer tissues (Uchida, *et.al.*, ١٩٩٩ and Zhao, *et.al.*, ٢٠٠٠), multidrug resistant cancer cells (Fiucci, *et.al.*, ٢٠٠٠) and transformed cell (Frankel, *et.al.*, ١٩٩٩). Demonstration of PLD production by the reverse CAMP test is useful in the identification of *A.haemolyticum* (Jurankova and Votava, ٢٠٠١).

Soucek, *et.al.*, (١٩٦٢) have reported that a substance in culture supernatant fluids inhibites the lytic action of beta-toxin of *Staphylococcus aureus*, and the description of the active substances in culture supernatant fluids is expanded to include three elements:

An (α) component possessed lecithinase activity, dermonecrotic activity and beta-hemolysin inhibiting activity, adsorbed to erythrocytes, and dissolved egg yolk (Soucek and Souckova, ١٩٦٦).

On the other hand (β) component lysed erythrocytes and (γ) component possessed lipase activity, possibly corresponding to phosphlipase A activity.

١.٢.٥.٢ Lipase

Lipases are enzymes that catalyze hydrolysis of fatty acid ester bond in tryiacylglycerol (TAG) thus releasing free fatty acid (note the reaction is reversible), So the enzyme can catalyze esterification of glycerol to form mono, di and triglyceride (Shalita, *et.al.*, ١٩٨٢).

Lipases have been reported to be large molecules of aggregates of protein- lipid or protein- protein (Pablo, *et.al.*, ١٩٧٤). Most lipases have optimum temperature ranging from ٣٠-٤٠C^{o}, but it should be recognized that while most enzymes stop catalysis at -١٠C^{o} because liquid substrates become solid ice.

Many lipases are produced constitutively, although their production may be influenced by the nutritional and physical condition of the culture (Kurioka and Matsuda, ١٩٧٥).

The physical role of extracellular lipase is probably nutritional; some may hydrolyze exogenous triglycerides to provide free fatty acids for use as an energy source (Lonon, *et.al.*, ١٩٨٨).

Lipase are important catabolic enzymes in phosphlipid metabolism; the mechanism of their action in infectious process is not well understood (Dennis, ١٩٨٣).

Lipase may help spread the microorganism in cutaneous and subcutaneous tissues (Koneman, *et.al.*, ١٩٩٢). Lipase that may be hydrolyzing lipid on epithelial surface of humans enhances the colonization of the skin (Willet, ١٩٧٦). The enhancement of growth by lipids by *Arcanobacterium* has been used as an aid to identification (Coyle, *et.al.*, ١٩٩٠).

١.٢.٥.٣ Deoxyribonuclease (DNase)

DNase is an extracellular endonuclease (Harper, *et.al.*, ١٩٧٧), that is specific for hydrolysis (degradation) of DNA . The physical and chemical properties of DNA differ from those of oligonucleotides or mononucleotides and these differences are used to detect hydrolysis of DNA by DNase (Blazevic and Edever, ١٩٧٥).

DNases are extracellular products that may serve to facilitate the liquification of pus and spreading of bacteria through tissue planes (Mandell, *et.al.*, ٢٠٠٠).

The *Arcanobacterium* DNase degrades host nucleic acid, making nucleotide available as nutrients (Jost and Billington, ٢٠٠٥).

The DNase of *Arcanobacterium* may aid in the depolymerization of higly viscous DNA released from disintegrating host cells in inflammatory lesions (Ramos, *et.al*., ١٩٩٧).

١.٢.٥.٤ Hemolysin

Hemolysin (bacterial) is a soluble substance elaborated by bacteria, that dissolves or breaks up red blood cell with consequent liberation of hemoglobin (Macfaddin, ٢٠٠٠). *A.haemolyticum* is considered to be beta- hemolytic, that hemolysis is far more subtle than what is seen with group A *Streptococcus* and can require ٤٨-٧٢ hours for detection (Cummings, *et.al.*, ١٩٩٣).

Colonies on sheep or human blood agar are circular discoid, opaque and whitish. Detection is clearly influenced by the choice of media and atmosphere of incubation (Cummings, *et.al.*, ١٩٩٣), both colony size and zone of hemolysis are significantly greater on tryptic soy agar (TSA) media. The CO_2 atmosphere is clearly superior for the production of larger hemolytic zones. Anaerobic incubation produces the smallest colonies and zone of hemolysis. Since the production of noticeable hemolysis is critical for the recognition of *A.haemolyticum* colonies in the presence of other flora (Cumming, *et.al.*, ١٩٩٣).

Trypticase soy agar is a superior media and CO_2 is a superior atmosphere for beta- hemolysis. The detection of hemolysis by *A.haemolyticum* can be facilitated by using special double – layered human blood agar plates (Bank and Nyman, ١٩٨٩).

١.٢.٥.٥ Neuraminidase

Neuraminidase is a potential toxin produced by *A.haemolyticum* (Muller, ١٩٧٣). Neuraminidase is a virulence factor, especially in bacteria that inhabit mucosal surfaces (Taylor, ١٩٩٦), and they may play several roles in virulence.

Neuraminidase (N- acetylneuraminyl hydrolase) removes sialic acid from glycolipid, glycoproteins, and poly- and oligosaccharides (Roggentin, *et.al.*, ١٩٨٩). This enzyme can make sialic acid a viable as a carbon source to promote growth in nutrient-limited environment (Byers, *et.al.*, ١٩٩٧). The action of neuraminidase can decrease mucous viscosity (Gottschalk, ١٩٦٠), possibly enhancing the colonization of the underlying tissues. The *Arcanobacterium* neuraminidases are probebly required for the colonization of the host and some (early) pathogenic processes, but may not be required in the latter stages of infections (Jost and Billington,٢٠٠٥). This enzyme is required for nasopharyngeal colonization and subsequent transition to the lower respiratory tract, but it is not required for lung infection (Orihuela, *et.al.*, ٢٠٠٤). Neuraminidase can increase the susceptibility of mucosal lgA to bacterial protease (Reinholdt, *et.al.*, ١٩٩٠).

Neuraminidase can enhance bacterial adhesion and colonization (Child and Gibbons, ١٩٩٠) as well as susceptibility of the host to the

action of toxins (Galen, *et.al.*, ١٩٩٢) by exposing cryptic host cell receptor molecules.

١.٢.٦ CAMP Test

This test is used to determine an organism´s ability to produce and elaborate the CAMP factor which acts synergistically with Staphylococcal beta- hemolysin (beta- lysine) on sheep erythrocytes to produce alytic phenomenon (Darling, ١٩٧٥). The CAMP factor is a thermo stable (heat- stable), extracellular (Christie, *et.al.*, ١٩٤٤), diffusible protein product of group B Streptococci (Brown, *et.al.*, ١٩٧٤), which lysis ruminant erythrocyte treated with beta-hemolysin (Jokipit and Jokipii, ١٩٧٦). It manifests specific synergism with sphingomyelinase C (Gubash, ١٩٧٨) on sheep blood. CAMP factor might interfere with precipitation of ceramide in situ; removal of ceramide would leave wide spaces in outer layer, rendering the inner layer in sheep cells susceptible to pressure from inside the cell and possible enzymatic attack or enough open areas in the outer layer of sheep to expose the inner layer to action of a broader-spectrum phospholipase C (Gubash, ١٩٧٨).

١.٢.٧ The CAMP Inhibition Test

CAMP inhibition (complete inhibition of the effect of *S.aureus* beta- hemolysin on sheep erythrocytes) is achieved by streaking the presumed *A.haemolyticum* strain at a right angle toward *S.aureus* and incubation overnight; beta- hemolysin inhibition zone in the form of triangle is observed, as in the case for *A. haemolyticum* (Patrick, *et.al.*, ٢٠٠٣).

The basis of the test is that *Staphylococcus aureus* and hemolysin act on the sphingomyelins in the cell membrane of sheep erythrocyte.

Beta-lysin of *Staphylococcus aurous* posseses phospholipase C (sphingomyelinase) activity (hemolysis, lysis) against sheep erythrocytes and hydrolyzes the sphingomyelin provided by the erythrocytes (Maheswaran and Lindorfer, ١٩٦٧).

Sphingomyelin (sphingolipids) is a class of lipids concerned with structure and function of cell membranes by providing barriers to indiscriminate exchange of compounds with extracellular fluids (Harrow and Mazur, ١٩٦٦).

Sphingomyelinase (sphingomyelin choline phosphohydrolase) from *Staphylococcus aureus* can attack the outer membrane of intact erythrocytes and produce water-insoluble ceramide and phosphorylcholine (Zwavall, *et.al*., ١٩٧٣). PLD hydrolyzes sphingomyelins to N-acylsphingosylphosphates (Souckov and Soucek,

١٩٧٤). *A.haemolyticum* phospholipase D (PLD) hydrolyzes these sphingomyelins, thus protecting the blood cell from haemolysis (Souckov and Soucek, ١٩٧٢).

١.٢.٨ Role of *A.haemolyticum in* pharyngitis

Pharyngitis, called sore throat, is the inflammation of the pharynx (throat). Acute bacterial pharyngitis is most often caused by haemolytic Streptococci; however, in acute pharyngitis of adolescents and young adult, *A.haemolyticum* can be isolated from about ٢٪ of the throat specimens (Bank and Nyman, ١٩٨٦). This organism has been reported to be associated with pharyngitis and systemic infections in immunocompetent adolescents and young adults (Alberto, ٢٠٠٣). *A.haemolyticum* has been increasingly identified as a cause of exudative pharyngitis, clinically simillar to that caused by beta-hemolytic streptococci (Karpathios, *et.al.*, ١٩٩٢).

Characteristically, the infections has been recognized in children, adolescents, and young adults and is associated with a diffuse, some times pruritic, erythematous maculopapular skin rash on the extermities and trunk. Cases of *A.haemolyticum* infections with membraneous pharyngitis that mimics diphtheria (Green and Lapeter, ١٩٨١) and with peritonsillar abcess have also been reported (Kovatch, *et.al.*, ١٩٨٣).

The clinical picture of *A.haemolyticum* pharyngitis is indistinguishable from that of Streptococcal pharyngitis (Waagner, ١٩٩١). Sore throat and pharyngeal erythema are always present.

Additional symptoms and signs include fever, non productive cough, skin rash, and tonsillar exudates. The exudates are gray or white and usually patchy in distribution, although they are sometimes confluent (Miller, *et.al.* ,١٩٨٦).

Lymphadenopathy is seen in ٢٥-٧٥٪ of the cases (Karpathios, *et.al.*, ١٩٩٢). Although the posterior aspect of the pharynx has a similar appearance to that in scarlet fever , associated hemorrhagic macules on the palate or findings on the tongue are not present (Miller, *et.al.*, ١٩٨٦).

The cutaneous manifestations appear unique to pharyngeal infections and have not been described in association with *A.haemolyticum* infections of other sites (Fell*, et. al.,* ١٩٧٧).

Sometimes skin rash has been the predominant symptom. The rash has been described only in patients with pharyngitis and not in patients with infections of other sites. The exanthema usually develops ١-٤ days after the pharyngitis, although occasionally, it is the initial manifestation of the infections. (Banck and Nyman, ١٩٨٦)

The rash is most prominent on extensor surfaces of the extremities but may extend centripetally to the chest and back.

Erythema multiform and urticarial rash have been described in patients with *A.haemolyticum* pharyngitis (Miller, *et.al.*, ١٩٨٦).

١.٢.٩ Antimicrobial Susceptibility

The antimicrobial susceptibility of *A.haemolyticumin* in vitro has been tested in only a few studies (Carlson, *et.al.*, ١٩٩٤). The organisms are usually susceptible to pencillin, and no strains resistant to erythromycin have been reported. *A.haemolyticum* has been reported to be uniformly susceptible to clindamycin, chloramphinicol, cephalosporins (Barker, *et.al.*, ١٩٩٢). Tetracyclines and aminoglycosides appear effective in vitro, although occasional resistant strains have been encountered (Worthington, *et.al.*, ١٩٨٥).

Tetracyclines are bacteriostatic, causing decrease in the effectiveness of penicillins when administered concurrently. Tetracycline acts by interfering with protein synthesis by binding to bacterial ribosome (Andrews, ١٩٩٠).This antibiotic is bacteriostatic especially in large doses. In smaller doses, oral antibiotics do not reduce the number of organisms but they affect their functions.

The antibiotics can also inhibit various enzyme activities (Webster, *et.al.*, ١٩٨٢), and modulate chemotaxic, lymphocyte function and proinflammatory cytokines (Eady, *et.al.*, ١٩٩٣). Tetracycline acts against susceptible microorganisms by inhibiting protein synthesis.

They enter bacteria by an energy- dependent process and bind reversibly to the ٣٠ S ribosomal subunits of the bacteria (Chopra and Roberts, ٢٠٠١). More often, resistance has been reported to sulphonamides,-trimethoprim (Ritter, *et.al.*, ١٩٩٣)

Although clinical *A.haemolyticum* isolates have been reported to be susceptible to vancomycin (Wickremesinghe, ١٩٨١), a few faecal isolates of *A.haemolyticum* are resistant and harboure the transferable vancomycin resistance gene Van A (French, *et.al.*, ١٩٩٢).

The susceptibilities of *A.haemolyticum* strains to cephalosporin, clindamycin, azithromycin and ciprofloxacin have not been reported before. But in a previous report all *A.haemolyticum* strains are susceptible to erythromycin (Bank and Nyman, ١٩٨٦).

No significant differences have been noted in the antimicrobial susceptibility between pharyngeal and extrapharyngeal clinical *A.haemolyticum* isolates (Waagner, ١٩٩١).

Macrolides also exhibit excellent activities against *Arcanobacterium* and are an alternative to B-lactam antibiotic for the treatment of infection, since treatment failures due to B-lactam antibiotic to act intracellular have been reported (Patrick, *et.al.*, ٢٠٠٣).

Reports of treatment failure with penicillin in spite of low minimum inhibitory concentrations have been attributed to

tolerance and to failure to penetrate the intracellular location of the pathogen. In most cases, large doses of intravenous penicillin G have been effective (Waller, *et.al.*, ١٩٩١).

There are only a few reports of the susceptibility of *A.haemolyticum* to antimicrobial agents and only the penicillin and erythromycin have been reported (Nyman, *et.al*., ١٩٩٠).

١.٢.٩.١ Penicillin VK

Oral penicillin has been studied and has been effective for treating pharyngitis, although some treatment failures have been reported (Karpathios, ١٩٩٢). The ability of *A.haemolyticum* to invade HEP-٢ cells and survive intracellular is investigated and has proved able to survive intracellular for ٤day thus creating intracellular reservoir of bacteria (Osterlund, ١٩٩٥). Although most of isolated strains are susceptible to penicillin, erythromycin, clindamycin and tetracycline, high doses of penicillin, with or without gentamycin, is recommended for the treatment of deep infections (Alonso*, et.al.,* ٢٠٠٢). In patients with bactermia, strains have been found to be resistant to penicillin, but clinical impact is unknown (Nyman, *et.al.,*١٩٩٠).

The bactericidal activity of the penicillins is often related to their ability to trigger membrane associated autolytic enzymes that desteroy the cell wall. This ability to inhibit bacterial cell wall

enzymes such as the transpeptidases usually confers on the penicillins bactericidal activity against gram- positive bacteria (Waxman and Strominger, ١٩٨٣). Penicillin inhibits biosynthesis of cell wall mucopeptide, which is bactericidal against sensitive organism when adequate concentration are reached and most effective during stage of active multiplication (Snow, *et.al.*, ٢٠٠١).

Reported successful treatment for systemic infections using a high dose of penicillin given intravenously erythromycin or penicillin in combination with aminoglycoside (Skov, *et.al.*, ١٩٩٨).

١.٢.٩.٢ Erythromycin

Erythromycin has been proposed as the drug of choice, with parenteral antimicrobial drugs used for serious infections (Gaston and Zurowski, ١٩٩٦).

A.haemolyticum seems to be uniformly susceptible to erythromycin in vitro. Erythromycin has been effective in oral doses of ٢٥٠ mg four times daily for seven days (Miller, *et.al.*, ١٩٨٦).

In children, age, weight and severity of infection determine proper dosage for more sever infections, dose is doubled. The strains is susceptible to erythromycin, ampicillin, vancomycin, clindamycin, rifampicin and penicillin. Erythromycin has been proposed as first-line therapy (Notario, *et.al.,* ٢٠٠١).

Erythromycin inhibits bacterial growth, possibly by blocking dissociation of peptidyl t RNA from ribosomes, arresting RNA-dependent protein synthesis (Miller, *et.al.*, ١٩٨٦).

Erythromycin has been effective and most strains are susceptible to this antibiotic.

Chapter Two

Materials and Methods

٢.١ Materials

٢.١.١ Laboratory Instruments

Table (١): Laboratory Instruments Used

No.	Instruments	Company
١	**Sensitive Electronic Balance**	A and D, Japan.
٢	Autoclave	Stermite, Japan.
٣	Incubator	Memmert, Germany.
٤	Distillator	GFL- Germany.
٥	Centrifuge	Hermle, Japan.
٦	Oven	Memmert, Germany.
٧	Refrigerator	Concord, Italy.

٨	Milipore Filter	Satorius membrane filter Gm bH, W. Germany.
٩	Light Microscope	Olympus, Japan.
١٠	Micropipette	Oxford, USA.
١١	pH Meter	Hoeleze and Cheluis, KG, Germany.
١٢	Water Bath	Memmert, Germany

٢.١.٢ Chemical and Biological Material

Table (٢): Chemical and Biological Materials Used

Name of material	Company
A- Chemical Materials	
Na_2HPO_4, K_2HPO_4, KNO_3, NaCl, $MgSo_4$, HCL, $CaCl_2$, KOH	Merk-Darmstadt.

alpha-nephthol amine, esculine, Tetramethyl-p-paraphylene diamine dihydrochloride, Acetic acid, Amyl alcohol, Sulfanilic acid, Methyl red, Olic acid, Chloroform, Phenol red, peptone	B.D.H
H_2O_2, Glucose, Sucrose, Trehalose, ٩٩٪ alcohol.	Fluka chemika-Switzerland
B- Culture media	
Blood agar base, Brain heart infusion agar, Brain heart infusion broth, agar-agar, Muller-Hinton agar	Mast.
Gelatin liquefaction medium, Pepton water medium, Urea agar medium, Nutrient agar media, Nutrient broth	Oxiod
Tryptic soy agar, Tryptic soy broth, Meet extract	Diffco-Michigan.

٢.٢ Patients and Methods

٢.٢.١ Patients

Three hunderd samples are collected from patients suffering from acute or chronic pharyngitis who admitid ENT unite in Babylon City

(children and adults) from both sexes. The history of disease is also obtained from each patient according to the following formula:

Name, Age, Sex, Chief Complaint and Duration, History of present illness, Drug history and history of systemic disease.

٢.٢.٢ Collection of Specimens

The specimens are generally collected from patients with pharyngitis. The swab should be used to collect as mush exudates as possible from the tonsils and posterior pharyngeal wall. The swab should be rubbed with rotatation over one tonsillar area, then the arch of the soft palate and uvula, the other tonsillar area, and finally the posterior pharyngeal wall. The swabs for culture should be placed in a tube containing normal saline to maintain the swab moist until it is taken to laboratory (Collee, *et.al*., ١٩٩٦).

The swabs are plated on a selective culture medium (Coman, *et.al.,* ١٩٩٦). (٥٪ sheep or human blood agar) using tryptic soy agar base and containing ٣.٥٪ NaCl. The plates are incubated for ٤٨- ٧٢ hours in an aerobic condition (٥-١٠٪ $Co_٢$) at ٣٧^{o}C.

This medium has been found to facilitate the isolation of *A.haemolyticum*.

٢.٢.٣ Reagents

١- Catalase reagent:-

It is prepared by adding ٣ml of H_2O_2 to ١٠٠ml distill water and stored in a dark container used to detect the catalase enzyme (Baron, *et.al*., ١٩٩٥).

٢- Oxidase reagent:-

This is soon prepared by dissolving ٠.١g of tetra-p-paraphenylene diamine dihydrochloride in ١٠ml of distill water and stored in a dark container (Baron, *et.al.,* ١٩٩٥).

٣- Nitrate reduction reagent:-

This is prepared according to (Collee, *et.al*., ١٩٩٦) .

The First solution is prepared by dissolving ٨g of sulfunilic acid in ١٠٠ml of ٥M acetic acid.

The Second solution is prepared by dissolving ٥g of alph-nephthyl amine in ١٠٠ml of ٥M acetic acid.

Equal volume of each solution is mixed to prepare the reagent.

٤-Kovac's reagent:-

This reagent has been prepared by dissolving ١٠gm from p-Dimethyl amino benzaldehyde (DAMB) in ١٥٠ml of amyl alcohol; ٥٠ml of concentrated HCL are gradually added to this mixture. This

solution is stored in a dark bottle, and gently shaken before use. It is used in the demonstration of indole production (MacFaddin, ٢٠٠٠).

٥-Phenol Red Reagent:-

It has been prepared by dissolving ٠.١g from phenol red stain in ٣٠٠ml of ethylalcohol (٩٥٪) then the volume is completed to ٥٠٠ml by distilled water. It is used to detect the acidity of the media ,which is produced by complete fermentation of carbohydrates (MacFaddin, ٢٠٠٠).

٦- Voges-Proskauer Reagent:-

A-٥g of alph-nephthol is dissolved in ١٠٠ ml of ٩٩٪ alchohol, and stored in a refrigerater in a brown glass bottle away from light.

B- ٤٠g of KOH is dissolved in ١٠٠ ml of distilled water (Collee, *et.al.,* ١٩٩٦).

٧- Methyl Red Reagent:-

It has been prepared by dissolving ٠.١g from methyl red stain in ٣٠٠ ml of ethyl alcohol (٩٥٪) then the volume is completed to ٥٠٠ ml by distilled water. It is used to detect the acidity of the medium,

which is produced by complete fermentation of carbohydrates (Collee, *et.al.,* ١٩٩٦).

٢.٢.٤ The preparation of media

١- Esculin Media:-

This media was made from preparation of nutrient agar with ١.٤gm ferric citrate and ٤gm easculin are added to it and volume was completed to ١٠٠ml; after that the media is poured into tubes and sterilized into autoclave, and then slant of media is formed (Baron, *et.al.,* ١٩٩٥).

٢- Lipolytic Media:-

The media contains the following ingredients;

٠.١g of peptone.

٠.٢٥g of sodium chloride.

٠.٠٠٥ g of calcium chloride.

٢ g agar.

All these are suspended in ٥٠ ml of distilled water, containing ٠.٥ml of oleic acid and this media was used to detect lipase production(Collee, *et.al*., ١٩٩٦).

٣- Nitrate Reduction Media:-

It has been prepared by dissolving a mixture of ٠.٢g of potassium nitrate (KNO_3), ٥g peptone in ٩٥٠ml of distilled water. In water bath, the final volume is distributed into test tubes and autoclaved. It is used for the detection of the ability of bacteria to reduce nitrate into nitrite (Collee, *et.al.,* ١٩٩٦).

٤- Peptone Water Media:-

It has been prepared by dissolving ٨g peptone in ١litter of distilled water, of distributed into test tubes, and autoclaved. It is used for the demonstration of the bacterial ability to decompose the amino acid treptophan to indole (MacFaddin, ٢٠٠٠).

٥- Gelatin Liquefication Media:-

It has been prepared by adding ١٢٪ gelatin to nutrient broth. This medium is used for testing the ability of bacteria to gelatin liquefaction (Collee, *et.al.,* ١٩٩٦).

٦- Urea Agar Media:-

This medium has been prepared by adding ١٥ml of urea solution (sterilized by filteration ٠.٤٥ mm) into ١٠٠ml of urea agar base sterilized by autoclaving and cooling to ٥٠C^o, the pH is adjusted into ٧.١ and the medium distributed into sterilized test tubes and allowed

to solidify in a slant form. It was used to test the ability of bacteria to produce enzyme urease (MacFaddin, ٢٠٠٠).

٧-Sugar Fermentation Media:

Medium is composed of:

A. Basal medium:

It is prepared by dissolving ١٠g pepton, ١g meat extract (Difco), ٥g Sodium chloride (NaCl) and ٠.٠٨ g phenol red (BDH) in one liter distilled water, then the pH is adjusted to ٧.٤. The Medium is distributed on test tubes, and durham tubes added to each test tube then sterilized by an autoclave (MacFaddin, ٢٠٠٠).

B. Sugar solutions:

Sugar solutions are prepared by dissolving ١g sugar in ١٠٠ml disteilled water and sterilized by filtration, then ٠.١ml sugar solution is add to each test tube (Item-١) containing ٥ml from the basal medium. The medium was used for diagnose pathogenic bacteria that have the ability to ferment sugar (MacFaddin, ٢٠٠٠).

٨- **M٩ media:-** (Miniatis, *et.al.,* ١٩٨٢)

٦g of Na_2HPO_4, ٣g of KH_2PO_4, ٠.٥g of NaCl, and ١g of NH_4Cl, are dissolved in ٩٥٠ml of D.W. with ٢٪ agar, and then sterilized into autoclave. After cooling the mixture to ٥٠^{o} C, ٢ml of ١m of $MgSO_4$, ١٠ ml of ٢٠٪ glucose and ٠.١ ml of ١m of $CaCl_2$ (sterilized them separately by filteration) are added, then the volume is completed to ١٠٠٠ ml .

٢.٢.٥ Biochemical Tests

١- Catalase Test:-

A small amount of bacterial growth on the medium at a ٢٤ hour age is transferred by sterile wooden stick onto the surface of a clean, dry glass slide, and one drop of (٣٪H_2O_2) is added to it. The formation of gas bubbles indicates the positive results (Collee, *et.al*., ١٩٩٦).

٢- Oxidase Test:-

A small portion of the colony to be tested is transferred by a sterile wooden stick to filter paper saturated with an indicator prepared soon. If the color around the smear turns rose to purple, the oxidase test is positive (Collee, *et.al*., ١٩٩٦).

٣- Esculin Test:-

The organism is grown in an esculin slant for ٢٤hr. at ٣٧°C. The dark brown color indicates a positive result. The unchanging of the color is a negative result (Baron, *et.al.,* ١٩٩٥).

٤- Indole Test:-

This test is performed by inoculating peptone water medium with bacterial growth, and incubated at ٣٧°C for ٤٨hr afterwards, ٠.٥ml of Kovacs reagent is added, and the tube was gently shaken; the formation of red- ring between the medium and reagents indicates a positive result (MacFaddin, ٢٠٠٠).

٥- Voges-Proskaure Test:-

The test is performed by using MR-VP broth. The inoculated media is incubated for ٢٤hr. at ٣٧°C. Afterwards, ١٥ drops of ٥٪ alpha-nephthol are added and followed by ١٠ drops ٤٠٪ KOH. The mixture is shaken well and allowed to stand up to ١٥ minutes before calling a reaction negative. If positive, the culture turns red at the surface of the liquid, and the color spreads gradually throught the

tube. The positive result indicates a partial analysis of glucose which produces (Acetyl-carbonyl) (Collee, *et.al.*١٩٩٦).

٦- Methyl Red Test:-

This test is carried out by inoculating tubes containing MR-VP medium with bacterial growth. The tube is incubated at ٣٧°C for (٢٤-٤٨) hr. and ٥ drops of methyl red indicator are added.The appearance of red color after ١٥ minutes indicates a positive test (Collee, *et.al.,* ١٩٩٦).

٧- Urease Test:-

This test is carried out by inoculating urea medium with bacterial growth and incubated at ٣٧°C for ٢٤h. The color change of medium into pink indicates a positive result (MacFaddin, ٢٠٠٠).

٨- Reverse CAMP Test:-

The CAMP inhibition test is achieved by streaking the presumed *A.haemolyticum* strain at right angle toward *Staphylococcus aureus* and incubation for ٤٨hr. beta – hemolysis inhibition zone in the form of a triangle is observed as in the case of *A.haemolyticum* (Patrick, *et.al.,* ٢٠٠٣).

٩- Carbohydrate Fermentation Test:-

Carbohydrate fermentation medium is inoculated with bacterial culture and incubated at ٣٧°C for ٢٤-٧٢ hr. The Color change of the

medium from red to yellow and gas formation in the Durham tube indicate a positive result (MacFaddin, ٢٠٠٠).

٢.٢.٦ Isolation and identification of *A. haemolyticum*

A.haemolyticum is slow growing beta-hemolytic Gram positive rod. The hemolysis and morphology of colonies are affected by the media, atmosphere and incubation time used for isolation.

Tryptic soy agar produces the largest colony size and incubation in carbon dioxide for minimum of ٤٨ hours yields the most discernible colonies (Cummings, *et.al*., ١٩٩٣).

The characteristic pitting of the agar may be observed by pushing the colony aside. Rough and smooth colony types have been described. Colonies are circular, discoid, opaque and whitish.

Further identification characteristics include a negative catalase and positive reverse CAMP test.

٢.٢.٧ Virulence Factors Tests

٢.٢.٧.١ Hemolysin production:-

Hemolysis production is shown on blood agar media. The results are obtained after the incubation of the non-cultured plates for ٢٤

hr. at ٣٧°C to exclude any contamination of blood, and then the organism is inoculated at this blood agar plates and incubated again for ٤٨ hr. at ٣٧°C. Any hemolysis presence should be detected around the colonies (Deboy, *et.al*., ١٩٨٠).

٢.٢.٧.٢ Lipase production:-

It is qualitatively prepared by incubating the isolates for ٢٤-٤٨ hours on lipolytic medium. Clearing of the medium around the colonies would indicate an isolate producing lipase (Chamberlain and Brueggemann, ١٩٩٧).

٢.٢.٧.٣ Protease production:-

M٩ is supplemented with ٢٪ agar used for the detection of protease enzymes. After sterilization in autoclave and cooling at ٥٠°C, ٠.٢٥ gm/L glucose (sterilized by filtrations) is added, and then the media is supported by ١٪ Gelatin. After the inoculation of this media with bacterial strain and incubation for ٤٨hr. at ٣٧°C, ٣ ml of Trichloroacetic acid (٥٪) is added to precipitate the protein. The positive result is read by observing a transparent area around the colony (Piret, *et.al*., ١٩٨٣).

٢.٢.٧.٤ Phospholipase D production:-

Brain heart infusion broth is prepared with ٠.٥٪ of glycerol. After sterilization in autoclave and cooling at ٥٠°C, the organism is inoculated at this broth and incubated again for ٤٨hr. at ٣٧°C.

The supernants fluids are then obtained by the filtrations of culture media containing *A.haemolyticum.*

Wells are made on Brain heart agar supplemented with ٥٪ sheep blood and then filled with filtrates to show the production of PLD.

Streaking of *Staphyloccus aureus* isolates are done horizontally by using sterile swab on all the surface of the medium except the area of the wells for ٤٨hr. at ٣٧°C.

The results show a beta-hemolysis inhibiting zone of *Staphylococcus aureus* around the wells.

٢.٢.٨ Effect of temperature on bacterial growth:-

The isolated bacteria are re-inoculated on blood agar and then incubated at various temperature degrees to show their ability to grow at these degrees.

٢.٢.٩ Antibiotic Sensivity Test

Antibiotic diffusion test (The Kirby-Baur susceptibility test) is used to show the effect of antibiotics on isolated bacteria.

١. It is performed by using a pure culture of previously identified bacterial organism.

The inoculums to be used in this test are prepared by adding growth from ٥ isolated colonies grown on blood agar plate to ٥ml of broth. This culture is then incubated for ٤٨hr. to produce bacterial suspension of moderate turbidity.

A sterile swab is used to obtain inoculums from the standardized culture. This inoculum is then streaked on tryptic soy agar plates supplemented with ٥٪ human blood.

٢. The antibiotic discs are placed on the surface of the medium at evenly spaced intervals with flamed forceps or disc applicator.

٣. Incubation for ٤٨hr. at ٣٧^{o}C, antibiotic inhibition zones are measured using caliper.

Zone size is measured to determine the susceptibility or resistance of organism to each antibiotic (MacFaddin, ٢٠٠٠).

Antibiotic disc supplied from Oxiod Company can be disc potency by mg / ml concentration as follows:

Antibiotics	Disc Potency
Ery.	١٥
Do.	٣٠
Ctx.	٣٠
Van.	٣٠
Cip.	٥
DA.	٥
Am.	١٠
Pn.	١٠
CN.	١٠
Azm.	١٥
Cl.	٣٠
Tet.	٣٠

Ery: Erythromycin; Do: Doxycyclin; CL: cephalexin; Ctx: cephotaxime; Van: Vancomycin; Cip: Ciproflaxine; DA: Clindamycin; Am: Ampicilin; Pn: Penicillin; CN: Gentamycin;

Azm: Azithromycin; Tet: Tetracycline.

Chapter Three

Results and Discussion

٣.١ Isolation and Characterization

This study deals with ٣٠٠ throat swabs obtained from patients (male or female) suffering from pharyngitis. The patient had been admitted to ENT unite Surgical Teaching Hospital in Hilla over period of ٨ month from October ٢٠٠٤ to May ٢٠٠٥.

All swabs have been subjected to culture on selective medium:

Sheep or human blood agar using tryptic soy agar base and containing ٣.٥٪ sodium chloride have been used to facilitate the isolation of *A.haemolyticum* and improve isolation of group A (*Streptococcus Pyogenes*) and group C and G beta-hemolytic S*treptococci*. commensal oropharyngeal flora such as viridance streptococci, *Neisseria* species, non haemolytic streptococci and beta- haemolytic streptococci not belonging to group A, B, C or G were significant inhibited (Watt, *et.al*., ١٩٩١).

It was seen that out of the total swabs obtained from patients with pharyngitis, ١٠٥ samples gave positive culture for *A.haemolyticum* and *Streptococcus sp.* and ١٩٥ samples did negtive culture, however, the results of bacterial isolation (Table ٣) show that only eight isolates (٢.٦٪) of *A.haemolyticum* were isolated.

Three of them from female patients and the other five isolates from male patients. This result is identical to those obtained by (Karpathios, *et.al*., ١٩٩٢) who have reported a higher incidence of pharyngitis caused by *A.haemolyticum* in males compared with females. But Fell, *et.al*., (١٩٧٧) have reported a higher incidence in female than in male.

The results obtained in this study are identical with those obtained by Linder, (١٩٩٧) who has succeeded to isolate *A. haemolyticum* at a rate reaching ٢.٥٪.

Bank and Nyman, (١٩٨٦) have also isolated *A.haemolyticum* from patients with pharyngitis at a rate of ٣٪ with the highest numbers among ١٥ – ٣٠ year old patients. Kuo, *et.al*. (١٩٩٥) and Roman and Garcia, (٢٠٠٥) have also reported the isolation of this bacteria from patients with pharyngitis by a rate of ٢.٥٪ and ٣٪ respectively, especially among adolescents.

In addition, Mackenzie, *et.al*. (١٩٩٥) have isolated *A.haemolyticum* from patients with pharyngitis at a rate of ٢.٥٪ with the maximum occurance in the ١٥ – ١٨ year-old age group.

Jurankova and Votava, (٢٠٠١) and Dorbat, *et.al*. (١٩٩٦) have isolated *A.haemolyticum* from patients with pharyngitis at a rate of (٠.٥٦٪) and (٠.٤٣٪) respectively. Arikan, *et.al*., (١٩٩٧) have also isolated *A.haemolyticum* from patients with pharyngitis at a rate of

٠.٣٪. Furthermore, Carlson, *et.al*., (١٩٩٥) and Chen, *et.al,* (٢٠٠٥) have isolated this bacteria by the rate of ١.٤٪ and ٠.٢٪ respectively.

The frequency of *A.haemolyticum* in throat swabs investigated is ٠.٠٧٪ as compared with *Streptococcus pyogenes* found in ٨.٢١٪ of cases (Coman, *et.al*., ١٩٩٦).

However, there are other types of bacteria than *A.haemolyticum* isolated in this study. All of these belong to the genus *streptococcus* sp.

It is known that oral cavity and most cases of phayngitis may be caused by *streptococcus* sp. ,so, the isolation of *Streptococcus* in this study is probable and is worth mentioning.(Table ٣).

Table (٣): Frequency of Positive Culture and Negative Culture on Selective Media

Patients No.	POSITIVE CULTURE		NE CU
	A.haemolyticum	*Streptococcus sp.*	
٣٠٠	٨	٩٧	
%	٢.٦٪	٣٢.٣٪	

Furthermore, the distribution of *A.haemolyticum* pharyngitis according to patient's ages has also been studied (Table ٤).

Most infections of *A.haemolyticum* appear at a high rate of (٧٥٪) among ages ranging from (٦ – ١٥) years old in both sexes and (٢٥٪) of the infection among the ages ranging from (٢٠ – ٤٠) years old. The results of this study are identical with those obtained by Arikan, *et.al*. (١٩٩٧) which deals with the prevelence of *A.haemolyticum* infections in the age between (٦ – ٢٢) years old. Kain, *et.al*., (١٩٩١) have also observed that *A.haemolyticum* infections are a common cause of pharyngitis in the ١٠ – ٣٠ year old age groups. However, pharyngitis caused by *A.haemolyticum* most commonly affects teenagers and young adults in the seconed and third decade of life (Carlson, *et.al*., ١٩٩٤).

Table (٤): Distribution of *A.haemolyticum* Infections among Age and Sex

Age	Sex		Number	%
	Male	Female		
١ _ ١٠	٢	١	٣	٣٧.٥
١١ _ ٢٠	١	٢	٣	٣٧.٥
٢١ _ ٣٠	١	٠	١	١٢.٥
٣١ _ ٤٠	١	٠	١	١٢.٥
> ٤٠	٠	٠	٠	٠
Total	٥	٣	٨	١٠٠

Moreover, there are no isolates of *A.haemolyticum* found in the healthy controls. This result is correlated with those results obtained by Fell, *et.al.*, (١٩٧٣) and David, *et.al*., (١٩٩٦) who have pointed that *A.haemolyticum* is not a part of the oral bacterial flora. On the other hand, Karpathios, *et.al*., (١٩٩٢) could not isolate *A.haemolyticum* from the throat flora of ٣٠٨ symptomless children. But Banck and Nyman, (١٩٨٦) have reported only one positive *A.haemolyticum* isolate among ٥٥٠ healthy control subjects.

All the patients who have positive culture for *A.haemolyticum* have sore throat and fever, non productive cough and skin rash.

The clinical picture in patients with *A. haemolyticum* pharyngitis is similar to that reported earlier except that the typical rash is detected less often.

This result is identical with that obtained by Carlson, *et.al*., (١٩٩٥) who have indicated that the typical rash is less often (٢٣٪) than in some other reports, ٤٦ – ٥٨٪ (Karpathios, *et.al*., ١٩٩٢).

٣.١.١ The Characteristics of *A.haemolyticum*

The definitive identification depends on the cultural characteristics and biochemical feature, the bacteria is gram – positive rod, catalase negative, facultivaly anaerobic bacteria and

reverse CAMP test (narrowing of the haemolytic zone produced by Staphylococcal of beta-lysine).

It is nutritionally fastidious and grow on complex media enhanced by contain a blood. *A. haemolyticum* produces acid from glucose, lactose and maltose (Goyal, *et.al.*, ٢٠٠٥).

On blood agar containing human blood, it forms colonies like streptococci surrounded by wide zone of beta-hemolysis (Collee, *et.al.*,١٩٩٦). *A. haemolyticum* produces opaque, whitish and tiny colonies (Waagner, ١٩٩١) figure (١). Rough and smooth colonies have been described (Fell, *et.al.*, ١٩٧٧). Most, but not all, strains produce a relatively beta-hemolysis.

The best medium for isolation and identification of *A.haemolyticum* is tryptic soy agar supplied with ٥٪ human blood and ٣.٥٪ NaCl. *A. haemolyticum* is a slow growing and the growth is enhanced by the addition of ٥ – ١٠٪ CO_2 (Cummings, *et.al.*, ١٩٩٣).

The most important cultural and biochemical characteristics of *A.haemolyticum* are summarized in Table ٥:

Table ٥: Diagnostic features of *A. haemolyticum*

Tests	Results
Growth on blood agar	White (smooth or rough biotype)
Shape of cell	Rod ,pleomorphic
Gram stain	Positive
Catalase	Negative
Indole	Negative
Oxidase	Negative
Nitrate reduction	Negative
Gelatin hydrolysis	Negative
Esculin	Negative
Type of haemolysis	Beta- haemolysis
Urease	Negative
VP	Negative
MR	Negative
Glucose	Positive
Maltose	Positive
Reverse CAMP	Positive

Figure (١)

***A. haemolyticum* appearance on blood agar**

٣.١.٢ Biotyping of *A.haemolyticum*

Colony morphology and beta hemolysis on sheep or human blood agar divide clinical *A. haemolyticum* isolates into smooth and rough

colony type (Carlson, *et.al*., ١٩٩٤). In this study, smooth and rough colony types are found on human blood agar after ٤٨hr. of incubation.

The smooth and rough colony morphologies correlate with two biotype. Each smooth colony has glistening surface and an entire edge surrounded by a zone of moderate to strong beta- hemolysis.

On other hand, each rough colony has rough surface and irregular edge. Beta hemolysis is either absent or very weak, and dark discoloration around the colony is frequently noticed. It has been observed that ٣(٣٧.٥٪) isolates are of the smooth type and ٥(٦٢.٥٪) are of the rough type.

This result is similar to those reported by (Carlson, *et.al*., ١٩٩٤) who have established that the smooth type at a rate of (٣٦٪) and rough type at a rate of (٦٤٪) from pharyngitis infections. The biotype could be distinguished using biochemical test described by (Krech and Hollis, ١٩٩١). The smooth type could ferment sucrose and trehalose while the rough type is negative for both sugars (Table ٦).

Table ٦: Properties of the two biotype of *A.haemolyticum*

Biotypes	No. of strain	Beta-hemolysis	Sucrose ferment	Trehalose ferment
Smooth	٣	Moderate	Positive	Positive

		to strong		
Rough	٥	Absent or weak	Negative	Negative

The rough biotype is isolated from respiratory tract specimens and the smooth biotype comes mainly from wounds. Whether this difference is due to, for instance, different adherence properties remains to be established (Carlson, *et.al*., ١٩٩٤).

٣.٢.٢ Reverse CAMP Test

The demonstration of phospholipase D (PLD) production by reverse CAMP test has been carried out (Zahorova and Kubella, ١٩٦٠). This test is useful in the identification of *A.haemolyticum*.

The results showed that the strains are positive for reverse CAMP which have the capacity to block the hemolytic activity of the beta-lysine (phospholipase C) produced by *Staphylococcus aureus* on sheep blood agar.

Beta-lysin of *Staphylococcus aureus* possesses phospholipase C (sphingomyelinase) activity (hemolysis, lysis) against sheep erythrocytes and hydrolyzes the sphingomyelin provided by the erythrocytes (Maheswaran and Lindorfer, ١٩٦٧).

Sphingomyelinase (sphingomyelin choline phosphohydrolase) from *Staphylococcus aureus* can attack the outer membrane of intact erythrocytes and produce water insoluble ceramide and phosphorylcholine (Zwaval, *et.al*., ١٩٧٣). PLD hydrolyzes sphingomyelins to N-acylsphingosylphosphates (Souckov and Soucek, ١٩٧٤).

The basis of the test is that *Staphylococcus aureus* and Hemolysin act on the sphingomyelins in the cell membrane of sheep or human erythrocytes.

A.haemolyticum phospholipase D hydrolyzes these sphingomyelins thus protecting the blood cell from haemolysis (Soucekov and Soucek, ١٩٧٢).

Bhat, *et.al*., (١٩٩٧) have showen that *A.haemolyticum* gives no hemolysis in CAMP test (reverse CAMP test positive) in contrast with CAMP tests which are used to produce CAMP factor which acts synergistically with *Staphylococcal* beta- hemolysin (beta-lysine) on sheep erythrocytes to produce alytic phenomenon (Darling, ١٩٧٥). The CAMP factor is extracellular diffusible protein produced by group B streptococci, which lysis ruminant erythrocyte treated with beta-hemolysin (Jokipit and Jokipii, ١٩٧٦).

٣.٣ Virulence Factors of *A.haemolyticum*

٣.٣.١ Hemolysin

Microorganism evolves a number of mechanisms for the acquisition of iron from their environments (Litwin and Calderwood, ١٩٩٣). One of them is the production of hemolysin which acts to release iron complexes to intracellular heme and hemoglobin. Iron can increase disease risk by functioning as a readily a vaible essential nutrient for invading microbial and neoplastic cell. To survive and replicate in hosts, microbial pathogens must a acquire host iron (Weinberg, ١٩٩٨).

To investigate the ability of *A.haemolyticum* to produce hemolysin on human blood agar, the results show that only three isolates are able to produce hemolysin and these isolates are smooth type (Table ٧). Whereas five isolates have no ability to produce this protein extracellulary and these isolates are rough types.

Carlson, *et.al*., (١٩٩٤) have shown that two biotypes of *A.haemolyticum:* one smooth type grows as a smooth, beta-hemolytic, whereas the other, the rough type, grows as rough colonies and is non hemolytic.

Furthermore, Hemolysin has also produced on tryptic soy agar supplemented with ٣.٥٪ NaCl and ٥٪ human blood. Both colony size and zone of hemolysis were significantly greater on tryptic soy agar media. The CO_2 atmosphere was clearly superior for production of

larger hemolytic zones. Anaerobic incubation produce the smallest colonies and zone of hemolysis since production of noticeable hemolysis is critical for the recognition of *A.haemolyticum* colonies in the presence of other flora (Cumming, *et.al*., ١٩٩٣). Tryptic soy agar is the superior media and CO_2 was the superior atmospere for beta-hemolysis.

٣.٣.٢ Protease

In the ability of *A.haemolyticum* to produce extracellular protease by using M_9 media (supported by ٠.٢٪ glucose and ١٪ gelatin) is investigated and it is found that all isolates are not able to produce extracellular protease after ٤٨ h. of incubation . There is no transparent area around the colony after the addition of ٣ml (٥٪) of trichloro acetic acid (TCA) (Table ٧).

This bacterium does not produce any secreated protease. It is defintely negtive for gelatin and casein hydrolysis (Parija, *et.al*.,٢٠٠٥), in contrast, *Arcanobacterium pyogenes* produce several protease which have the capacity to degrade host proteins releasing amino acid as nutrients and may degrade proteins such as IgA which are involved in host defense, and may also be involved in host tissue damage (Jost and Billington, ٢٠٠٥). These bacteria closely

resemble *Arcanobacterium haemolyticum* but *Arcanobacterium pyogenes* hydrolyse gelatin rapidly.

٣.٣.٣ Lipase

The ability of *A.haemolyticum* to produce lipase has been investigated. All isolates are able to produce lipase after ٤٨hr. of incubation (table ٧). This result is identical with those obtained by (Coyle and Lipsky, ١٩٩٠) who have pointed that this bacteria produce lipase.

The role of lipase in pharyngitis infections is not known. There is no previous study to indicate the role of this enzyme in *Arcanobacterium haemolyticum* infections in respiratory tract. However, the role of this enzyme may have a role in cutaneous infections as conflect mentions by (Al-khafaf, ٢٠٠٥).

The majority of microbial lipase has been reported to be large molecules of aggregates of protein-lipid or protein-protein (Pablo, *et.al*., ١٩٧٤). A structure of this type explains some of the confecting data on the size and specify lipase as described by various groups isolated from different growth phases (Pablo, *et.al*., ١٩٧٤).

In cutaneous infections, this enzyme may help spread the organism in cutaneous and subcutaneous tissue through hydrolyzing

lipid on epithelial of surface of humans, enhanced colonization of the skin

(Koneman, *et.al*., ١٩٩٢).

The enzyme production can be arrested by various compounds such as tetracyclines which are effective against lipase production by interfereing with protein synthesis by binding to bacterial ribosome (Andrews, ١٩٩٠).

٣.٣.٤ Phospholipase D

The ability of *A.haemolyticum* to produce phosphlipase D (PLD) has been investigated. All isolates are able to produce PLD after ٤٨ h. of incubation (table ٧). The supernant fluied of PLD inhibites the lytic action of beta- toxin produced by *Staphylococcus aureus* (Figure ٢). Cuevas and Songer, (١٩٩٣) have reported to produce soluble toxins including PLD and suggested a role of these enzymes in pathogenesis of infections by *A.haemolyticum.*

The PLD gene from *A.haemolyticum* has been cloned and shown to have a high degree of homology with that of *C.pseudotuberculosis* where it is thought to participate in vascular permeability and dissemination of the pathogen (Cuevas and Songer, ١٩٩٣). PLD is an essential virulence determinant that contributes to the persistence and spread of the bacteria within the host (Mcnamara, *et.al*., ١٩٩٥).

PLD is the main virulence factor for this bacterium which catalyzes the hydrolysis of phosphatidylcholine (PC), the major membrane phospholipid, to form phosphatidic acid (PA) which may promote the colonization of the bacteria to the tissues and then induction of hemolytic activity and apoptosis (Mcnamara, *et.al*., ١٩٩٥).

These enzymes have broader substrate specificity, and their ability to catalyze transphosphatidylation reactions has made them useful for synthesis of un natural lipids (Mcdermott, *et.al*., ٢٠٠٤). PLD is known to bring about tissue damage as elaborated by *A.haemolyticum* as well as the closely related bacterium, *Corynebacterium pseudotuberculosis* an impotant pathogen of sheep (Linder, ١٩٩٧). The role of potentiated cytotoxicity caused by the combined activity of PLD and cooperative agents such as cholesterol data involving in cophagocytosis (Linder and Bernheimer, ١٩٨٢).

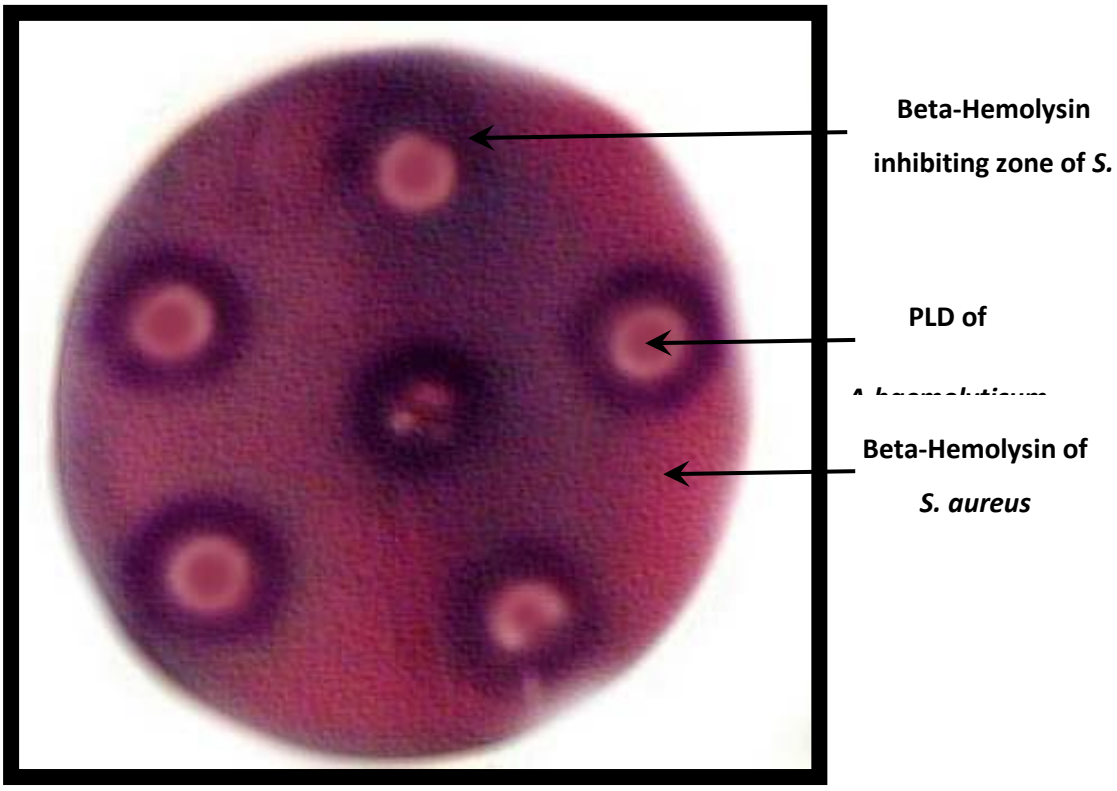

Figure (٢)

Phospholipase D Production by *A. Haemolyticum*

Table (٧): Detection of virulence factors on the *A. haemolyticium*

Virulence factors	Isolation No.							
	١ (rough)	٢ (rough)	٣ (rough)	٤ (smooth)	٥ (rough)	٦ (rough)	٧ (smooth)	٨ (smooth)
Hemolysin	-	-	-	+	-	-	+	+
Protease	-	-	-	-	-	-	-	-
Lipase	+	+	+	+	+	+	+	+
PLD	+	+	+	+	+	+	+	+

PLD: phospholipase D

٣.٤ Effect of Temperature on *A.haemolyticum* Growth

As shown in Table (٨) the results reveal that *A.haemolyticum* grows well at (٤٠-٤)^{o}C and that the optimum growth of *A.haemolyticum* isolates at temperature ranging from ٣٥-٣٧^{o}C under anaerobic conditions which resembles the temperature of the body.

The bacterial growth has been completely inhibited at a temperature of ٤٥^{o}C. The results are identical to those recorded by

Waagner, (١٩٩١) who showed that the Optimum growth of *A.haemolyticum* occurs at ٣٧°C, in either microaerphilic or anerobic conditions. The temperature can effect the bacterial enzymes through it is influence on the three- dimensional configuration of protein, thereby affecting the rates of enzymatic activities.

The optimal temperature of growth is ٣٧ºC but a culture can be obtained for other tempertures (in particular for tempertures ranging between ٢٠ and ٤٠ºC (Euze'by, ١٩٩٩).

Table (٨): Effect of Temperature on the Growth of *A. haemolyticum* Isolates

Temp. °C	Iso.١ (rough)	Iso.٢ (rough)	Iso.٣ (rough)	Iso.٤ (smooth)	Iso.٥ (rough)	Iso.٦ (rough)	Iso.٧ (smooth)	Iso.٨ (smooth)
٤٠ – ٤	+	+	+	+	+	+	+	+
> ٤٠	–	–	–	–	–	–	–	–

٣.٥ Effect of some Antibiotics on *A.haemolyticum*

Some antibiotics are used to show their effect on *A.haemolyticum* isolates such as erythromycin, deoxycycline, cephalexin, cefotaxime, vancomycin, ciprofloxacin, clindamycin, ampicilin, pencilin, gentamicin, azithromycin and tetracycline .

It has been found that *A.haemolyticum* isolates are sensitive (١٠٠٪) to erythromycin, penicillin and azithromycin and resistant (١٠٠٪) to gentamycin whereas some isolates have shown resistance in lesser degree to vancomycin (٦٢.٥٪), cefotaxime (٥٠٪), cephalexin, clindamycin and ampicilin (٣٧٪). The isolates are also resistante to doxycycline, ciproflaxine and tetracycline at a rate of (٢٥٪) (Table ٩).

Table ٩: Effect of antibiotics on the Growth of *A .haemolyticum*

Isolate No.	Ery.	Do	Cl.	Ctx.	Van.	Cip.	DA.	Am.	Pn.	CN.	Azm.	Tet.
١ rough	-	-	+	-	+	+	-	+	-	+	-	-
٢ rough	-	-	+	+	-	-	+	-	-	+	-	-
٣ rough	-	+	-	+	-	-	-	+	-	+	-	-
٤ smooth	-	+	+	-	+	+	+	-	-	+	-	+
٥ rough	-	-	-	-	+	-	-	-	-	+	-	-

٦ rough	-	-	-	+	+	-	-	-	-	+	-	-
٧ smooth	-	-	-	+	-	-	+	-	-	+	-	+
٨ smooth	-	-	-	-	+	-	-	+	-	+	-	-
Rate of resistant %	٠	٢٥	٣٧.٥	٥٠	٦٢.٥	٢٥	٣٧.٥	٣٧.٥	٠	١٠٠	٠	٢٥

+: Resistant, -: Sensitive

There are only afew reports of the susceptibility of *A.haemolyticum* to antimicrobial agents and only the penicilin and erythromycin have been reported (Nyman, *et.al.,*١٩٩٠).

All the isolates have shown sensitivity to penicillin. This result is identical with those obtained by (Cambier, *et.al*., ١٩٩٢) and (Carlson, *et.al*., ١٩٩٤). However some reports, have mentioned that treatment failure with penicillin has been attributed to tolerance and to failure to penetrate the intracellular location of the pathogen (Linder, ١٩٩٧).

The ability of *A.haemolyticum* to invade HEP-٢ cells and survive intracellularly has been investigated. (Alos, *et.al*., ١٩٩٥) and (Skov, *et.al*., ١٩٩٨) have also reported that most isolates are considered to be tolerant to penicillin. On other hand, some isolates have shown resistance to ampicillin. Notario, *et.al*., (٢٠٠١) have observed that the isolates of *A.haemolyticum* are susceptible to ampicilin.

Furthermore, all the isolates have shown sensitivity to erythromycin and this antibiotic, as it is known is the drug of choice for *A.haemolyticum* treatment (Gaston and Zurowski, ١٩٩٦).

Erythromycin is known as a macrolied antibiotic and is the first choice for patients with penicillin alleragies a ١٠ day regimen is needed (Bisno, ٢٠٠١).

Erythromycin, an antibiotic known to penetrate well intracellularly, efficiently kills these bacteria (Osterlund, ١٩٩٥). This antibiotic inhibits bacterial growth, possibly by blocking dissociation of peptidyl-tRNA from ribosomes, arresting RNA-dependent protein synthesis (Miller*, et.al*., ١٩٨٦).

On the other hand, all the isolates have shown sensitivity to azithromycin. Another macrolide, azithromycin can be given as using daily dose and may be effective in five days. This antibiotic is bacteriostatic agent which inhibit the growth of microorganisms by binding to the ٥٠S subunit of the prokaryotic ribosome, blocking protein synthesis at the peptidyltransferase step.This antibiotic is likely efficacious for *A.haemolyticum* (Donald and Middleton, ١٩٩٦). But this antibiotic produces fewer gastrointestinal side effects than does erythromycin (Michael and Pichichero, ١٩٩٨). Macrolids also exhibit excellent activities against *Arcanobacterium* and are an alternative to beta-lactam antibiotic for the treatment of infection,

since treatment failures due to beta-lactam antibiotic to act intracellular have been reported (Patrick*, et.al*., ٢٠٠٣).

The results also show that only two isolates of *A.haemolyticum* are resistant to tetracycline. This result is similar to those obtained by (Carlson, *et.al*., ١٩٩٥). The Two strains belong to smooth biotype and their resistance to this drug is most commonly mediated either by active efflux of tetracycline from the cell or by ribosomal protection from the action of tetracycline Chopra and Roberts, (٢٠٠١). Probably the presence of TetW gene is widespread determinant of tetrcycline resistnce in *Arcanobacterium* sp. (Billington, *et.al.,* ٢٠٠٢).

Tetracycline is bacteriostatic especially in large doses and it is short acting. In small doses oral antibiotics do not reduce the number of organisms but they affect their function. The antibiotics can also inhibit various enzyme activities such as lipase by interfering with protein synthesis combining with bacterial ribosome (Andrews, ١٩٩٠).

Tetracyclines are incompletely absorbed from the gastrointestinal tract, but their absorption is improved in the fasting state.Food-mediated interference with absorption is lower with doxycycline .The results also show that two isolates are resistant to doxycycline.These antibiotics long – acting and more readily absorbed; therfore;lower doses are required (Chopra and Roberts, ٢٠٠١).

Five isolates of *A.haemolyticum* are found resistant to vancomycin. The high-level constitutive resistance seen in these isolates appears to be mediated by Van A genes (Power*, et.al*., ١٩٩٥). The isolates of *A.haemolyticum* have previously been shown to be resistant to vancomycin and to harbour the transferable vancomycin resistance gene Van A (French, *et.al*., ١٩٩٢).

Besides, the effect of clindamycin on *A. haemolyticum* isolates was studied and it has been found that three isolates are resistant to clindamycin; two strains belong to the smooth biotype. Clindamycin inhibits bacterial growth possibly by blocking dissociation of peptidyl tRNA from ribosoms causing RNA- dependent protein synthesis to arrest (Tai and Daris, ١٩٨٥). This result is similar to those obtained by (Carlson, *et.al*., ١٩٩٩).

Furthermore, all isolates of *A.haemolyticum* are resistant to gentamycin. This results contrasts with those obtained by Nyman, *et.al*., (١٩٩٠) who have observed that the isolates of *A.haemolyticum* are sensitive to gentamycin .

Aminoglycosides appear effective invitro, although occasinol resistant strains have been encountered (Worthington, et.al., ١٩٨٥).

The results also show that only two isolates of *A.haemolyticum* are resistant to ciproflaxein. This antibiotic can inhibit bacterial DNA synthesis, an event that is followed by rapid bacterial cell death.

Carlson, *et.al.,* (١٩٩٤) have reported that the isolates are senstive to ciproflaxin.

The results also show that three isolates (٢٥٪) are resistant to cephalexin. Biswas, *et.al*., (٢٠٠٣) have found that the isolates of *A.haemolyticum* are senstive to cephalexin. This antibiotic is the first-generation and cephalosporin has broader spectrum agents for all sore throat which arrests bacterial growth by inhibiting bacterial cell wall synthesis. Cephalosporins are potent, and are very effective in eradicating the bacteria. The results also show that some isolates are resistant to cefotaxime. It is a synthetic, broad-spectrum cephalosporin antibiotic. The bactericidal activity of cefotaxime results from the inhibition of cell wall synthesis (Mary, *et.al.,* ٢٠٠٠).

Goyal, *et.al*., (٢٠٠٥) have observed that *A.haemolyticum* are sensetive to cefotaxime.

However, there is no difference between smooth and rough biotype in response to antibiotic sensitivity test, except for some antibiotic such as clindamycin and tetracycline. The results show that some strains belonging to smooth biotype are resistante to these antibiotics. These differences are qualitative but not essential.

Briefly, *A.haemolyticum* has recently been accepted as an important human pathogen but has been reported infrequently as a cause of well – defined infections, probably because of failure to

correctly identify the pathogen in clinical specimens. The correct identification and antibiotic sensitivity testing of such isolates are essential for the proper management of infected individuals.

Chapter Four
Conclusions and Recommendations

٤.١ Conclusions

١. *A.haemolyticum* could easily be isolated on selective media using sheep blood agar with tryptic soy agar base containing ٣.٥٪ sodium chloride.

٢. The number of *A.haemolyticum* isolated from ٣٠٠ patients suffering from pharyngitis (children and adults) from both sexes is eight isolates (٢.٦٪).

٣. Cultures for streptococcal pharyngitis will allow detection of *A.haemolyticum*: when this bacteria is searched for, blood agar plates must be incubated for full ٤٨hr. and examined carefully for typical haemolytic colonies.

٤. A sufficient number of subcultures and Gram stains should be performed. Especially if haemolytic streptococci are not detected in patients suffering from pharyngitis.

٥. The isolates are susceptible to penicilin, cephalosporins, macrolids, doxycyclin, clindamycin and vancomycin, but resistant to gentamycin.

٦. Two strains of *A.haemolyticum* were isolated rough and smooth biotypes on the basis of colony morphology and biochemical tests.

٧. *A.haemolyticum* is revealed to posseses more than one virulence factor such as phospholipase D, lipase, hemolysin production.

٨. Reverse CAMP test has been used for the identification of this bacteria. This test is useful for demonstrating PLD production by *A.haemolticum.*

٤.٢ Recomendations

According to the results obtained in the present study, it is recommended that further investigation be made as follows:

١. Study the prevelance of *Arcanobacterium haemolyticum* in cases other than pharyngitis.
٢. Investigations of other species of *Arcanobacterium* to show their prevelence in Iraq.
٣. Study of molecular basis of *Arcanobacterium haemolyticum* isolates in various infections.
٤. Detection on other enzymes and proteins associated with the pathogenicity of these bacteria.

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