2-Aminoacetophenone As A Virulent Factor For Pseudomonas Aeruginosa Causing Sever Burn And Wound Infections In Iraq

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Abstract

Various pathological specimens (180) were collected from patients suffering from pseudomonas aeruginosa infections from different hospitals in Baghdad from January to May 2011; these specimens include (Blood samples,sputum,urine and wound swabs) were tested for pseudomonas aeruginosa producing 2-Aminoacetophenone.Wounds swabs specially taken from burns and post surgical infections producing a higher concentration of 2-Acetophenone material than from other samples were tested for this material and most of these were isolated bases on their distinctive grape- like odor of 2-Aminoacetophenone production usually linked with patients whose immune system compromised by disease or trauma, its gains access to these patients tissue through burns, although the grape odor is sometimes difficult to detect in culture media.

These methods can be utilized to assay 2-aminoacetophenone (2AA) production in a varies media. Its synthesis occurs relatively early in the growth cycle. It has proved easy and convenient to detect 2-aminoacetophenone (2AA) excretion by Pseudomonas aeruginosa after 24 h of incubation on blood agar plates employing thin layer chromatography of ether extracts of the agar medium.

Keyword: 2-Aminoacetophenone , Pseudomonas Aeruginosa ,gramnegative Baeteria.

Introduction

pseudomonas aeruginosa is a gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium that has an incredible nutritional versatility. It is a rod about 1-5 µm long and 0.5-1.0 µm wide. P. aeruginosa is an obligate respirer, using aerobic respiration (with oxygen) as its optimal metabolism although can also respire anaerobically on nitrate or other alternative electron acceptors.

p. aeruginosa is an opportunistic human pathogen. It is "opportunistic" because it seldom infects healthy individuals. Instead, it often colonizes immunocompromised patients, like those with cystic fibrosis, cancer, or AIDS [1]. It is such a potent pathogen that firstly, it attacks up two thirds of the critically-ill hospitalized patients, and this usually portends more invasive diseases. Secondly, P.aeruginosa is a leading Gram-negative opportunistic pathogen at most medical centers, carrying a 40-60% mortality rate. Thirdly, it complicates 90% of cystic fibrosis deaths; and lastly, it is always listed as one of the top three most frequent Gram-negative pathogens and is linked to the worst visual diseases [2].

It also exhibits intrinsic resistance to a lot of different types of chemotherapeutic agents and antibiotics, making it a very hard pathogen to eliminate [3].

P. aeruginosa was first described as a distinct bacterial species at the end of the nineteenth century, after the development of sterile culture media by Pasteur. In 1882, the first scientific study on P. aeruginosa, entitled "On the blue and green coloration of bandages," was published by a pharmacist named Carle Gessard. This study indicated that P.

aeruginosa's characteristic pigmentation: P. aeruginosa produced water-soluble pigments, which, on exposure to ultraviolet light, fluoresced blue-green light. This was later attributed to pyocyanine, a derivative of phenazine, and it also reflected the organism's old names: Bacillus pyocyaneus, Bakterium aeruginosa, Pseudomonas polycolor, and Pseudomonas pyocyaneus [3].

P. aeruginosa has many strains, including Pseudomonas aeruginosa strain PA01, Pseudomonas aeruginosa PA7, Pseudomonas aeruginosa strain UCBPP-PA14, and Pseudomonas aeruginosa strain 2192 [4]. Most of these were isolated based on their distinctive grapelike odor of aminoacetophenone, pyocyanin production, and the colonies' structure on agar media [5]. Since P. aeruginosa can live in both inanimate and human environments, it has been characterized as a "ubiquitous" microorganism. In addition, P. aeruginosa is an opportunistic human pathogen that causes chronic infections in patients with cystic fibrosis and is the leading cause of death by Gram-negative bacteria [1].

Although most P. aeruginosa-plant interactions are detrimental to the plant, a recent study has found a P. aeruginosa strain that actually supports plant growth. This characteristic, along with the fact that P. aeruginosa can degrade polycyclic aromatic hydrocarbons, suggests the future uses of P. aeruginosa for environmental detoxification of synthetic chemicals and pesticides and for industrial purposes [1].

P. aeruginosa rarely causes disease in healthy humans. It is usually linked with patients whose immune system is compromised by diseases or trauma. It gains access to these patients' tissues through burns, for the burn victims, or through an underlying disease, like cystic fibrosis. First, P. aeruginosa adheres to tissue surfaces using its flagellum, pili, and exo-S; then, it replicates to create infectious critical mass; and lastly, it makes tissue damage using its virulence factors [6]. Since the powerful exotoxins and endotoxins released by P. aeruginosa during bacteremias continue to infect the host even after P. aeruginosa has been killed off by antibiotics, acute diseases caused by P. aeruginosa tend to be chronic and life-threatening. And even though a small amount of patients infected by P. aeruginosa developed severe sepsis with lesions with black centers, most patients exhibited no obvious pathological effects of the colonization [6].

P. aeruginosa secrets many virulent factors to colonize cells of its host. For example, exotoxin A, the most toxic protein produced by P. aeruginosa, catalyzes the ADP-ribosylation to form ADP-ribosyl-EF-2, which inhibits the protein synthesis of the host's cells. Epidermal infections often result from P. aeruginosa infiltrating through a human host's first line of defenses, entering the body through the skin at the site of an open wound. P. aeruginosa is a common member of hospital bacterial communities where it can infect immunocompromised individuals including burn victims. P. aeruginosa is a source of bacteremia in burn victims [7] Following severe skin damage, the prevalence of P. aeruginosa in the environment increases the probability of the organism accessing the bloodstream through the burn victim's exposed deep epidermal tissue[7]. The pili and flagella of P. aeruginosa play a vital role in the infection of burns and wounds [7].

The spread of P. aeruginosa within host organisms is also dependent on the microorganism's elastase production and other protease mechanisms. Bacterial elastase and other bacterial proteases degrade the host's proteins, including the structural proteins within membranes, disrupting the host's physical barriers against the spread of infection. The virulence in P. aeruginosa is both combinatorial and multifactorial and that the genes required for one strain to be pathogenic are not required for virulence in other strains [11].

Materials and Methods

Bacteria and culture conditions

Bacteria were obtained from different pathological specimens collected from patients of the Baghdad Medical city and three other hospitals in Baghdad from January to May 2011 and inoculated into media directly from initial blood agar plates usually maintained on brain heart infusion agar (Difco) with monthly transfers .Rich media were tryptone, Trypticase soy broth, brain heart infusion, meat infusion (Difco).Casamino acids and yeast extract (Difco) media in 1% concentrations supplemented with 0.4 mM Mgcl$_2$ [11]. Minimal media consisted of 10 mM concentrations of substrates, 0.4 mM Mgcl$_2$, 0.1 mM K$_2$SO$_4$,4 mM NH$_4$Cl and 4 mM potassium phosphate buffer at PH 7.4.The medium used for the analysis of 2AA production consisted of 10 Mm tryptophan, 0.1% yeast extract, 0.4 mM MgCl$_2$ and 4 mM potassium phosphate buffer at PH 7.4.Tryptophan was sterilized by filtration through filter with 0.45-um pore size (Millipore Corp,) and added to the yeast extract medium after autoclaving. Blood agar plates consisted of blood agar base medium (Difco) plus2% sheep erythrocytes (20 ml per plate) .[12]

Identification of 2AA

P. aeruginosa microorganism was grown in 1 liter of a medium containing 0.1% yeast extract, 4 mM potassium phosphate buffer (PH 7.5), 0.4 mM MgCl2 and 10mM tryptophan at 37°C with shaking for 24 h. The cells were centrifuged (3000 rpm/min.) from the medium, and the supernatant fluid was extracted with 100 ml of ether in a separatory funnel [13].The ether layer was removed and reduced to 0.5 ml by passing nitrogen gas over the surface. The ether solution was dried with anhydrous magnesium sulfate and injected onto a 10% SP1000 column in a Varian Aerograph series 2700 gas chromatography equipped with a flame ionization detector [14]. The elution of the grape odor from the column was matched to a peak on the recording by noting the time the odor was detected at the ejection port. The compound was then collected from subsequent injections by trapping the compound from the ejection port in a glass capillary tube chilled with dry ice. The trapped compound was dissolved in pentane and injected onto an SP 1000 Colum in a gas chromatograph [15].

Identification of the compound responsible for the grape odor of p. aeruginosa as 2AA was based upon three criteria. The compound from ether extracts of culture media migrated at the same location as authentic 2AA on thin-layer chromatograms.

Time course of 2AA and quinazoline synthesis during culture

It was important for physiological and diagnostic reasons to know the time of synthesis of 2AA during the growth curve. p. aeruginosa microorganisms was inoculated into 1 liter of a solution containing 0.1 % yeast extract, 10 mM tryptophan, 4 mM potassium phosphate buffer PH 7.4, 0.4 mM MgCl2 and 10 uM FeCl3 at a density of 10^3 bacteria per ml. Growth was measured by the optical density at 650 nm by using 1-cm cuvettes in a Gilford model 240 spectrophotometer. A various times during incubation, samples of 50 ml were withdrawn and extracted with 20 ml of ether.

The layers were concentrated and analyzed by gas chromatography. The culture reached a maximum optical density after 30 h. The 2AA concentration reached maximal level at approximately 22 h. The concentration of 2AA then decreased rapidly to 40 uM by 80 h of culture. Repetitions of the experiment using both gas chromatographic and thin-layer chromatography yielded the same pattern of 2AA.

Time course of 2AA synthesis during culture without tryptophan

To determine the time of synthesis of 2AA in a medium similar to the rich media used for clinical isolation, a l% solution of brain heart infusion broth was prepared with 0.4 mM MgCl$_2$ and a microorganisms of p. aeruginosa was inoculated at a density of 10^2 bacteria per ml. Growth was followed by the optical density of the culture at 600 nm and 40 ml samples

were removed at various times for extraction into ether. The ether layers were concentrated and processed for quantization of 2AA.The culture reached the stationary phase after approximately 20 h of incubation, while the concentration of 2AA in the medium showed a burst of 2AA synthesis at 16 h followed by substantial increase in 2AA concentration as the culture entered the stationary phase [20].

Production of 2AA in various media

The effects of different culture media on thproduction 2AA were determined by inoculating 10^3 bacteria per ml into 40 ml of 1% Solutions of rich media in 250 ml flasks which were incubated with shaking at 37"C.After 20 h of incubation, the cultures were centrifuged to remove the cells, and the spent media were adjusted to pH 10 and extracted with 20 ml quantities of ether. The ether layers were concentrated and analyzed by chromatography on silicic acid thin layers.

Analysis of the production of 2AA on blood agar by clinical isolates

Blood agar is often used as a primary isolation medium for pathogenic bacteria and is constructed with a base medium containing a high concentration of beef heart infusion or meat infusion, which as shown in (table-2), allows considerable production of 2AA by p. aeruginosa isolated from burn unit; all of these bacteria are pyocin production. They were individually streaked for isolation on blood agar plates. The plates were incubated at 37 C for 20 h and were extracted by flooding a plate with 5 ml ether. The plate was swirled occasionally for 5 minutes and then the ether was transferred by pipette to a test tube. This procedure can be used with plastic plates. No pH adjustment is required since blood agar after incubation with p. aeruginosa becomes alkaline. The most direct method of detecting 2AA in the extracts is by viewing the tubes under a handheld ultraviolet lamp (360 nm). Most tubes demonstrate very strong fluorescence, visible even in a well-lighted room, whereas other shows a more moderate fluorescence, still easily distinguishable in a dark room.

Assay of 2AA

Two methods were used to assay 2AA in the ether extracts of culture media [15].The extraction procedure consisted of mixing 50 ml of culture medium which had been adjusted to pH 10 with 20 ml of ether in a 250 ml separatory funnel. The ether layer was removed and concentrated to between 1 and 4 ml by applying a vacuum (aspirator) to a desicator containing the extracts. Gas chromatographic assay of 2AA was conducted in a Varian Aerograph series 2700 gas-liquid chromatograph on columns' of Carbowax 20 M, quantitation was accomplished by injection increasing concentrations of 2AA, cutting the peaks corresponding to 2AA from the chromatographs, and comparing the weights of the peaks with the quantities of authentic 2AA injected. The standard curve was linear from 0.5 to 10 ug. Thin- layer chromatograms of authentic 2AA and ether extracts of alkaline culture media yielded single yellow spots at the position of 2AA when the developed chromatograms were sprayed with Ehrlich reagent (1 gm of p-dimethylaminobenzaldeyde (sigma) in a solution containing 25 ml of HCl and 75 ml of methanol) [16]. Colorimetric assays for 2AA were conducted on 2 ml samples of ether extracts of alkaline culture media by adding 1 ml of 1% p-dimethylaminobenzaldeyde in methanol, followed by 2 ml of glacial acetic acid. After 10 minutes, the absorbance at 475 nm was compared with a standard curve of absorbance versus 2AA concentration. This curve was used in the range 1 to 8 umol/ml of 2AA .The spectrum of the chromophor was determined in a perkin Elmer model 124 spectrophotometer, and the routine colorimetric assays were conducted with a Gilford model 240 spectrophotometer [17].

Results

A- Identification of 2AA

Chromatographic Gas analysis of authentic 2AA and the bacterial product on two chromatography support material gave identical retention times (table-1). Analysis of the bacterial grape odor compound yielded a fragmentation pattern confirming its identity as 2AA[18].

B- Time course of 2AA and quinazoline synthesis during culture

Quinazolines were not detected early in culture. Only after 40 h was there sufficient 4-methylquinazoline to allow quantitation.2,4-Dimethylquinazoline never accumulated in high concentrations, but a concentration of 1 uM 4-methylquinazoline was attained at 60 h of culture [10,19].

C- Production of 2AA in various media

Brain heart infusion and peptone media allowed the highest levels of 2AA production, yeast extract and meat infusion media allowed intermediate levels and casamino acids, tryptone and trypticase soy broth media allowed low levels of 2AA accumulation (table-2). Growth on tryptophan as a substrate allowed the greatest accumulation of 2AA of any of compounds tested in minimal medium, but growth on this substrate was extremely slow and a 48 h incubation period was necessary to obtain maximal growth and a yield of 0.68 uM 2AA.Because the biosynthetic pathway for 2AA branches from the tryptophan catabolic pathway, tryptophan was incorporated into various growth media in 10 mM concentrations. This evoked an increase in the2AA concentration found in the cultures even those in minimal media (table-2).

D- Analysis of the production of 2AA on blood agar by clinical isolates

Thin-layer chromatography of extracts of p. aeruginosa demonstrated that fresh extracts from plates incubated for 20 h contained 2AA as the only detectable fluorescent compound. Other fluorescent copounds were detectable in minute amounts after aging the extracts or extracting plates which had been incubated for 48 h or longer.

Discussion

It is the most common cause of infections of burn injuries and of the outer ear (otitis externa), and is the most frequent colonizer of medical devices (e.g., catheters). Pseudomonas can, in rare circumstances, cause community-acquired pneumonias, [21] as well as ventilator-associated pneumonias, being one of the most common agents isolated in several studies [22]

Pyocyanin is a virulence factor of the bacteria and has been known to cause death in Caenorhabditis elegans by oxidative stress. However, research indicates that salicylic acid can inhibit pyocyanin production [23].

One in ten hospital-acquired infections is from Pseudomonas. Cystic fibrosis patients are also predisposed to P. aeruginosa infection of the lungs. P. aeruginosa may also be a common cause of "hot-tub rash" (dermatitis), caused by lack of proper, periodic attention to water quality. The most common cause of burn infections is P. aeruginosa. Pseudomonas is also a common cause of postoperative infection in radial keratotomy surgery patients. Pseudomonas aeruginosa is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with P. aeruginosa via the foam padding found in tennis shoes, with diabetic patients at a higher risk Burn wound infection is problematic because it delays healing, encourages scarring and may result in bacteremia, sepsis or multiple-organ dysfunction syndrome (organ failure) whereby organs from several systems are unable to maintain homeostasis on their own, requiring immediate medical attention [24].

Two bacterial species, methicillin-resistant staphylococcus aureus (MRSA) and Pseudomonas areuginosa will be examined in depth in this page as they are two of the most prevalent infective agents. These two species have proven particularly difficult to treat because they posses a large number of virulence factors and antimicrobial resistance genes. P. aeruginosa are the most common source of burn infections [24]. They are Gram-negative bacilli that possess a single supercoiled circular chromosome. Pseudomonas is also a common cause of postoperative infection in radial keratotomy surgery patients. The organism is also associated with the skin lesion ecthyma gangrenosum.

Pseudomonas aeruginosa is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with P. aeruginosa via the foam padding found in tennis shoes, with diabetic patients at a higher risk [25,26]. The chromatography confirmed that 2AA is responsible for the grape odor of p. aeruginosa. Two other products, 4-methylquinazoline and 2,4 dimethylquinazoline were separated and identified along with 2AA by gas chromatography [27,28].

The quinazoline are produced in low concentrations late in the stationary phase of growth and do not interfere with the assay for 2AA.Growth studies indicate that 2AA is an early product during the growth cycle, accumulating in large amounts after 20 to 30 h of culture. Although the amount of 2AA produced at 20 to 24h is not necessarily represents the maximal concentrations, this is an incubation period which is convenient for rapid analysis in clinical laboratories. Although addition of tryptophan to media increases the production of 2AA, it is not necessary for the production of large amount in rich media. Olfactory detection of 2AA is often used as a diagnostic aid in identifying p. aeruginosa and the nose is usually sensitive to levels lower than 1 ug/ml. Olfactory sensation rapidly becomes saturated with 2AA , however, interfering with repeated analyses.

There may also be many interfering odors in the media or other odors produced by the organisms. This report offers the colorimetric assay for rapid detection and quantization of 2AA.The detection of fluorescence in the extracts with hand-held ultraviolet lamp is the most sensitive method. The colorimetric assay may be conducted on the entire ether extract to aid in the identification of the fluorescence as due to 2AA.The ability to measure 2AA in alkaline extracts depends upon the lack of charge on the molecule at basic PH values which allows extraction of 2AA into the ether phase free from other fluorescing molecules. This rapid assay may be of use in early diagnosis of p. aeruginosa on plates used for primary isolation. It may also be of use on plates containing mixtures of microorganisms, but caution must be used because it is not known whether other bacteria produce fluorescent molecules of similar charge which could interfere with the assay. This paper presents information on 2AA production in culture and techniques for identification which may aid in analysis of the compound responsible for the grape odor of p. aeruginosa. 2AA is generated from tryptophan during autoclaving and uninoculated media yield varying amounts of 2AA from tryptophan depending upon aeration, temperature and exposure to light during incubation. Control must be included during short as well as prolonged incubation to assess the nonenzymatic degradation of tryptophan.

Detection of 2AA and the grape odor is a supplement to other characteristics used to identify p. aeruginosa. Clearly much more information must be obtained about this compound and its production by other bacteria before an assessment of its importance in diagnosis may be made. P. aeruginosa infections are difficult to treat because these microbes possess multiple strategies for eluding predators, including multidrug efflux pumps, antibiotic-modifying enzymes, and tough outer membranes with low permeability [28].

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Table(1): Gaschromotographic separation of 2AA from various pathological specimens

Infection	No.of sample	Retention time (ml/min)SP1000	With Carbowax 20M
Bacteremia	11	24	8
Burn wound	37	28	6
Lung (pneumonia)	13	26	5.8
U.T.I.(Cathetrization)	55	16	31.2
Ear(Otitis external)	10	10	30
Respiratory tract(diffuse bronchopneumia)	22	22	7.8
Bones and Joints(Vertebral osteomylatis,pyoarthrosis,sternoclavicular joint)	12	25	8.1
Skin(soft tissue, haemorrhage and necrosis)	6	18	11.2
Septic shock(Associated with apurple-black skin lesion)	14	25	7.6

Table(2): Production of 2AA by Pseudomonas aeruginosa in various media

Infection	No. of Sample	Medium	Concentration of 2AA	
			With Tryptophan	Without Tryptophan
Bacteremia	11	Peptone	100	120
		Brain Heart Infusion	60	210
		Glycerol	0.05	5
		Trypticase soys broth	0.4	22
Burn wound	37	Peptone	88	16
		Brain Heart Infusion	52	190
		Glycerol	0.02	6
		Trypticase soys broth	0.3	20
Lung	13	Peptone	88	18
		Brain Heart Infusion	52	190
		Glycerol	0.02	6
		Trypticase soya broth	0.3	20
U.T.I	55	Peptone	60	8
		Brain Heart Infusion	55	110
		Glycerol	0.02	6
		Trypticase soya broth	0.2	10
Ear	10	Peptone	40	6
		Brain Heart Infusion	22	64
		Glycerol	0.02	3
		Trypticase soya broth	0.2	1
Respiratory tract	22	Peptone	82	18
		Brain Heart Infusion	62	200
		Glycerol	0.05	6
		Trypticase soya broth	0.3	18
Bones and Joints	12	Peptone	78	20
		Brain Heart Infusion	56	186
		Glycerol	0.04	5
		Trypticase soya broth	0.4	20
Skin	6	Peptone	80	20
		Brain Heart Infusion	66	190
		Glycerol	0.04	5
		Trypticase sota broth	0.3	20
Septic shock	14	Peptone	92	22
		Brain Heart Infusion	62	210
		Glycerol	0.05	3
		Trypticase soya broth	0.3	12

إعتبار ثنائي أسيتومينوفيون عامل شدة مرضية متولد نتيجة لاصابة الجروح والحروق الشديدة بجرثومة pseudomonas aeruginosa في العراق

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الخلاصة

أجري الفحص لـ 180 أنموذجاً لحالات مرضية لأشخاص يعانون من إصابة الجروح والحروق بالتلوث بجرثومة الزائفة الزنجارية والمأخوذة من مختلف مستشفيات بغداد، وقد وجد أن النماذج كانت تحتوي على دم، قشع، إدرار ومسحات من جروح وحروق ملوثة بتلك الجرثومة.

لوحظ أن هذه الجرثومة تولد مادة ثنائي أسيتومينافتون بكميات أكثر عند اصابتها للحروق والجروح الملتهبة، اذ إن هذه المادة لها رائحة تشبه رائحة العنب عند زرعها على مختلف الاوساط الزرعية.

مما تقدم يمكن أعتبار تلك المادة عامل شدة مرضية خاصة بتلك الجرثومة الزائفة الزنجارية التي تسبب التهابات شديدة للجروح والحروق.

الكلمات المفتاحية: أمينو2- أسيتومينوفيون، Pseudomonas Aeruginosa، بكتريا اكرام سالب