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Study Synergistic Interactions between Antibiotics and Ascorbic Acid against *Pseudomonas Aeruginosa* Isolated Bovine Mastitis

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Abstract

The present study was carried out to isolate *Pseudomonas aeruginosa* from cattle (bovine) milk with mastitis and shed light upon some materials that worked in increasing antibiotic activity against isolated bacteria. Two hundred and twenty bovine milk samples were collected randomly from different local cow farms at districts of Wasit governorate, Iraq. During the period from the mid of October 2021 to the end-February 2022.37(16.8%) P. aeruginosa isolates were obtained using bacterial culture method and further identified by Analytical Profile Index (API-20E) and Vitek 2 system. Antibiotic susceptibility test was conducted for bacterial isolates , tested by disc diffusion method using (12) antibiotics and the results showed a different percentage of resistance to each antibiotic as (Gentamycin, amikacin, ampicillin, bacitracin, Ciprofloxacin, Norfloxacin, chloramphenicol, erythromycin, tetracycline, streptomycin, tobramycin, Trimethoprim_sulfamethoxazole). The results revealed that Ciprofloxacin was the most effective antibiotic against bacterial isolates followed by amikacin and then by Norfloxacin, and the isolates are completely resistant to both erythromycin and tetracycline. Twelve isolates were selected to detect the effect of ascorbic acid when was combined with antibiotics and tested by using disk diffusion assay. Various concentrations of the ascorbic acid were used, starting from (1 to 22.2 mg). The results showed that there is a synergistic interaction between vitamin C and most of the antibiotics, Also, the synergistic effect increases with increasing concentration of the vitamin. The antibiotic chloramphenicol had the greatest effect, as the area of inhibition increased in 11 out of 12 isolates. Also, the tests showed that ascorbic acid had an antagonistic effect on some antibiotics such as norfloxacin and tobramycin, where the inhibition area decreased in 9 and 8 isolates, respectively.

Keywords: *Pseudomonas aeruginosa*, Ascorbic acid, Bovine Mastitis.

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is one among the world's top ten superbugs, causing diseases in humans and animals (1). Therapeutic options are still severely limited due to the emergence of antimicrobial-resistant strains; hence, infection with P. aeruginosa remains a life-threatening concern (2). Serious infections, both acute and chronic, are always nosocomial and linked to weakened host defenses; however, this opportunistic pathogen is increasingly being identified as the cause of disease in livestock and companion animals, including otitis and urinary tract infections in dogs and cats, mastitis in dairy cows, sheep, and goats, hemorrhagic pneumoniae in mink cows and goats, hemorrhagic pneumoniae in mink and foxe Mastitis caused by P. aeruginosa can affect dry cows or animals that have recently given birth. The high incidence of fecal carriage of this organism can contaminate agricultural water supplies, and the presence of particular forms of pyocin in the udder, stomach, or water can lead to transmission from one reservoir to another.to another; however, many types did

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not appear to spread [3].Antibiotic-resistant bacteria are on the rise across the world, posing a serious threat of treatment failure [4]. Ascorbic acid has antibacterial, antiviral activity and is essential to stimulate the immune function (activate phagocytic leukocytes) [5], decrease the severity and duration of an infection [6] and reduce the inflammation that resulted in bacterial infection [7]. [8] Claimed that large dosages of ascorbic acid worked synergistically with suitable antibiotics when used to treat bacterial infections, significantly broadening the drugs' action range.

Methods

Samples collection.

A total of 180 bovine milk samples, irrespective of age or season, were taken aseptically and directly from the udder in sterile cups from mastitic and apparently healthy cows from different local cow's farms in different districts of Wasit governorate. The specimens were gathered between October 2020 and the end of April 2021.

Bacterial identification

overnight, the milk samples were incubated at 37 degrees Celsius. The cells were then cultivated on nutritional agar, LB agar, and MacConkey's agar (HiMedia/India) and incubated aerobically for 24-48 hours at 37 °C. The colonies that were suspected were picked up and streaked on Cetrimide agar.; and was identified by biochemical tests, including: oxidase test (Fluka/Switzerland), API 20NE kit (BioMerirux/ UK) (12) and andVitek 2 system.

Antibacterial agents

Twelve different forms of antibiotics were used, described in Table (2.1).

Table (1): List of antibacterial agents used in sensitivity testing.

Antibacterial agents	codes	concentration Mg/ disk	Antibacterial agents	codes	concentration Mg/ disk
Gentamycin	GM	10	Chloramphenicole	C	30
Amikacin	AK	30	Erythromycin	E	15
Ampicillin	amp	10	Tetracycline	TE	30
Bacitracin	BA	10	Streptomycin	S	10
Ciprofloxacin	CIP	5	Tobramycin	TOB	30
Norfloxacin	NOR	10	Trimethoprim-sulfamethoxzole	SXT	5

Antibacterial susceptibility test

This test was done according to the procedure defined by Murray, et al., (2007); twelve different forms of antibiotics were used, described in Table (1) above, The disk diffusion test (DDT) was employed to assess the antimicrobial susceptibility of P. aeruginosa isolates.

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Twelve different antibiotics were used, as listed in Table (1). According to the guidelines of [10], sensitive and resistant isolates were found.

Determining Effect of Ascorbic acid in Combination with Antibiotics

Different molarities of ascorbic acid solution were prepared to start from (22.2 mg), and the lowest molarities of ascorbic acid that causes inhibition against bacteria were determined by using a diffusion assay. The solution of Ascorbic acid was prepared and the desired concentration of antibiotics was added. The paper disk was socked in the final solution, and disk diffusion assays were used to determine the inhibition zone of antibiotic disks against bacteria according to the Clinical And Laboratory Standards Institute [10].

Statistical Analysis.

The Chi-square test was used to statistically analyze all of the data using the system SPSS IBM version 20 program. Statistical significance was defined as a P-value of less than 0.001 [11].

Results and discussion

Prevalence of *Pseudomonas aeruginosa* in milk samples

Out of 220 simples 37 (16.8%) was The Prevalence of *Pseudomonas aeruginosa* **in** cattle (bovine) milk with mastitis samples.The isolates grew faster on LB agar at 37 ℃ and appeared as convex, smooth, non-lactose fermenting colonies with regular margin and pale color. On MacConkey agar and nutrient agar, these bacteria appeared smooth at fresh isolation, converted to mucoid spreading growth due to bacterial swarming, with conversion of almost dish to the greenish color or without greenish pigment production in some isolates; some isolates produced water-soluble greenish pigment on nutrient broth . P. aeruginosa isolates differ from other species of Pseudomonas by growth in selective medium (Cetrimide agar (Figure .3-1). The bacterial colonies on Cetrimide agar were seen as convex, smooth at fresh isolation, and then converted to mucoid distinguished in their color and spreading growth .then further identified by Analytical Profile Index (API-20E) and Vitek 2 system.

Figure (1):. Growth of *P. aeruginosa* onCetrimide agar

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Mastitis is probably the most important health disorder on dairy farms. This is reflected in relatively high incidence of clinical mastitis and on many farms a high prevalence of subclinical mastitis. In case of Pseudomonas, mastitis is only sporadic, but occasionally it may be a serious herd problem, and udder infection is usually regarded as an opportunist, being relatively non-invasive and producing disease more often after injury of debilitating conditions, or secondary to other infectious agents. Also, the use of common or non sterile teat cannulas for intramammary administration of antibiotics have been involved in the introduction and spread of Pseudomonas mastitis [12]. The milk samples collected from cattle in the present study revealed presence of P. aeruginosa in 16% of the cases. Such isolation of P. aeruginosa was recorded recently by other workers such as [13] in which 30 milk samples were taken from milk of cattle infected with mastitis fromdifferent fields in Al-Diwanyia province [14].recorded that contamination of raw cow milk and soft cheese samples with P. aeruginosa in Baghdad was (76.7%); (19) isolated 10%; (20) isolated 3.0%; (21) isolated 6.9% and (22) reported 3.6% isolation in mastitis cows.

Antibiotics susceptibility testing for Pseudomonas aeruginosa

Antibiotics are being tested resistance in microorganisms is critical for classifying their actions based on the types of antibiotics used, as well as their medicinal use and efficacy in disease care. Furthermore, it will provide a visual representation of the subsequent transmission of genetic elements responsible for resistance across species and, as a result, the identification of resistance spread [15].The requisite test for screening purposes should be capable of detecting a large number of isolates. The disk diffusion method (used in this study) is quick and simple, but it has a lower degree of accuracy since the zone of inhibition is influenced by the medium structure and interaction of certain ions with antibiotics distributed through the medium [16].Thirty-seven isolates were subjected to susceptibility testing according to the CLSI , 2020 [10] guidelines using different antibiotics namely (Gentamycin, amikacin, ampicillin, bacitracin, Ciprofloxacin, Norfloxacin, chloramphenicol, erythromycin, tetracycline, streptomycin, tobramycin, Trimethoprim-sulfamethoxazole) the test show at the figure (2,3).

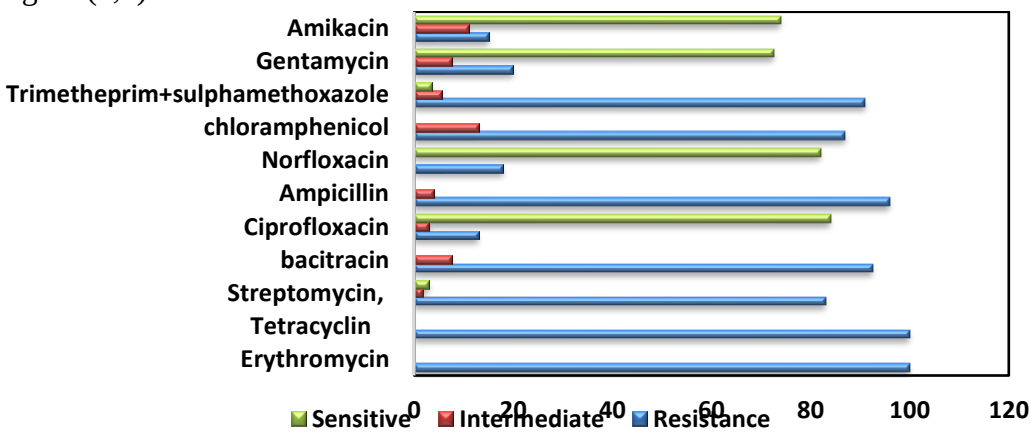

Figure (2): The percentage of antimicrobial resistance profiles of *pseudomonas aeruginosa*

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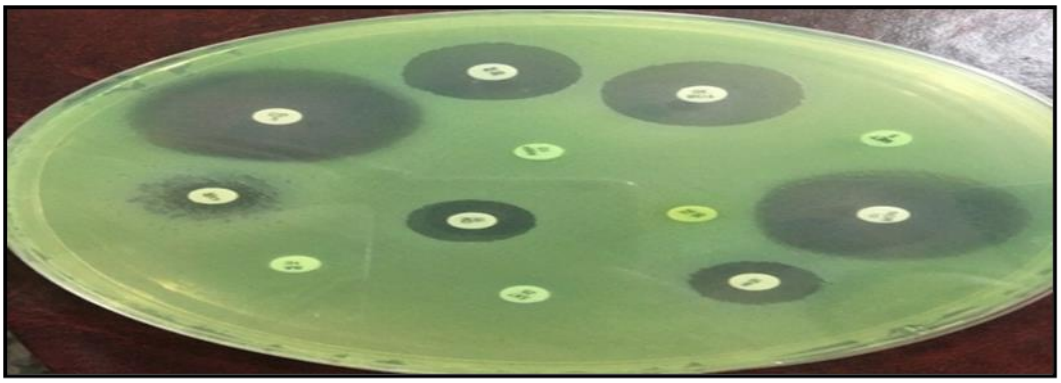

Figure (3): Sensitivity test by using discs diffusion assay.

The unselective use of antibiotics is potentially leading to a higher incidence of infections with resistant microorganisms such as P. aeruginosa. Regarding, the antimicrobial sensitivity agar it was noticed that the isolates were lack of susceptibility to many tested antimicrobial agents. The primary mechanism of the microorganism's resistance relies on its ability to shut out various agents rather than the production of antibiotic inactivating enzymes. Consequently, most antibiotics are of limited value in the treatment of P. aeruginosa infection [17]. The results indicated that the isolates are completely resistant to both erythromycin and tetracycline (100%). These findings agreed with [18,19] were reported the P. aeruginosa is completely resistant to erythromycin and tetracycline, Resistance to erythromycin may be due to the common use of this antibiotic which leads to increase microbial resistance to this antibiotic, while resistance to Tetracycline may be due to the resistance gene carried by plasmid [20]. In this study, isolates showed high resistance to ampicillin (96.29%). This result agrees with the results obtained by [21] who found that Ps. aeruginosa was completely resistant to ampicillin (100%), while [33] revealed that Ps. aeruginosa was resistant to ampicillin at 89.9%. Regarding streptomycin, all bacterial isolates showed high resistance percentage to it (83.33%). This result disagreed with those of [19] who reported high resistance to Ps. aeruginosa(90%). Resistance to gentamycin recorded a low resistance percentage. The Percentage of resistance was (20.37%). [22], reported gentamycin sensitivity for Ps. Aeruginosa 40%, 30% respectively. Ciprofloxacin on the other hand had shown a good effect on the bacterial isolates. Most isolates were found to be sensitive; for example, ciprofloxacin sensitivity was 87% for Ps. aeruginosa, This result was almost comparable to the results reported by [23] who found that ciprofloxacin sensitivity of Ps. aeruginosa was (86%). Sensitivity of bacteria to ciprofloxacin (quinolones) because quinolones act principally by inhibiting bacterial DNA Gyrase, so preventing supercoiling of the DNA, a process that is necessary to compacting chromosome into the bacterial cell. Results showed high resistance to erythromycin with (100%). While [24] mentioned that Ps. aeruginosa resistance to erythromycin was (69.5%). Resistance to Trimethoprim-sulphamethoxazole "SXT" were (90.74%). [19] found the same result as the SXT resistance to Ps. Aeruginosa (100%). The Percentage of resistance to Tobramycin was (24%). [25] reported different findings, the resistance percentage was (80.7%) in Baghdad and disagreed with [23] in Iraq, who recorded

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percentage resistance with (57.2%). Amikacin percentage resistance in this study was (14.81%). This result disagrees with the study by [26] result was (34.9%).

Enhancement of antibiotics activities

Effect of ascorbic acid in combination with antibiotics

The susceptibility testing of *P. aeruginosa* against antibiotics alone and their combination with ascorbic acid was checked by the disc diffusion method. Combination discs of antibiotic and ascorbic acid in different concentrations (1,6.4.13.8.22.2 mg) were prepared and their efficacy against *P. aeruginosa* was checked by measuring their zone of inhibition around disks, in addition to using several methods of adding acid to the disks of antibiotics, such as the pouring method and the method of pipette addition Synergistic effect were produced when antibiotic and ascorbic acid use together. It was observed that the activity of antibiotics was greatly enhanced and the resistance of bacteria was reversed with ascorbic acid. Total 12 samples of *P. aeruginosa* were checked for their susceptibility against antibiotics and antibiotic+ Ascorbic acid combination.

The results showed that ascorbic acid increases the zone diameters of Ampicillin, Tetracycline, and Bacitracin 83.33 % (10 of 12) of the isolates, while Amikacin and Ciprofloxacin appeared a positive effect on half of the samples and a negative effect on the other half. The synergic effect also recorded with using Gentamicin and Erythromycin appeared in 7 out of 12 (58.33%) of the samples. Regarding chloramphenicol, it had a significant positive activity on 11/12 (91.66%) of the samples. Furthermore, Norfloxacin had inhibitory activity on 9/12 (75%) of isolates, unlike streptomycin and Trimethoprim-sulfamethoxazole, it had a synergistic effect on the same number of isolates. , while for tobramycin, it had an interaction effect, as the inhibition zone was reduced in 8 out of 12 (66.66%) of the bacterial isolates at different concentrations Figure (3-4,5,6).

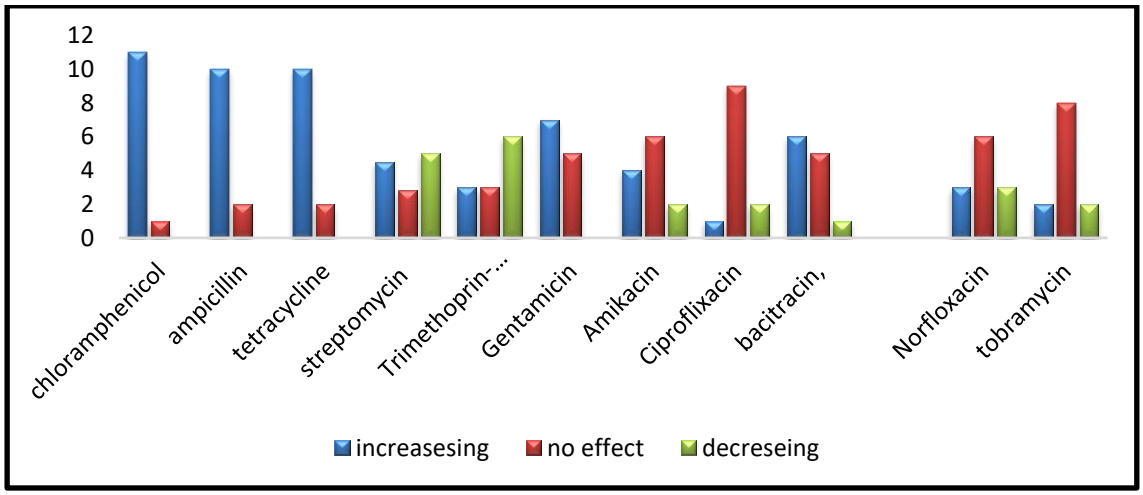

Figure (4): Effect of Antibiotic ascorbic acid combination on Zone of inhibition diameters.

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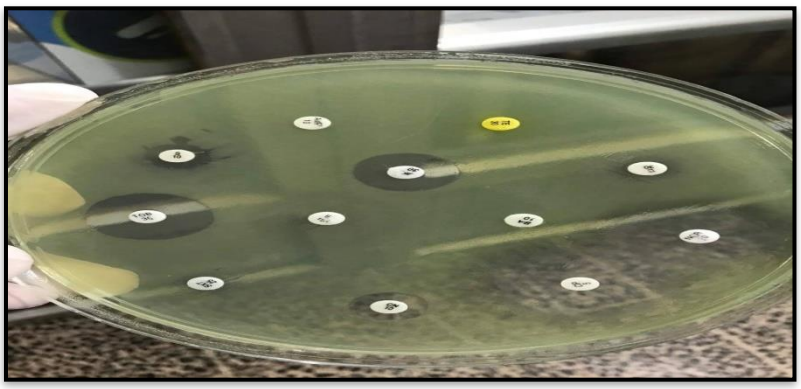

Figure (5): The effect of antibiotic and ascorbic acid (6.3 mg).

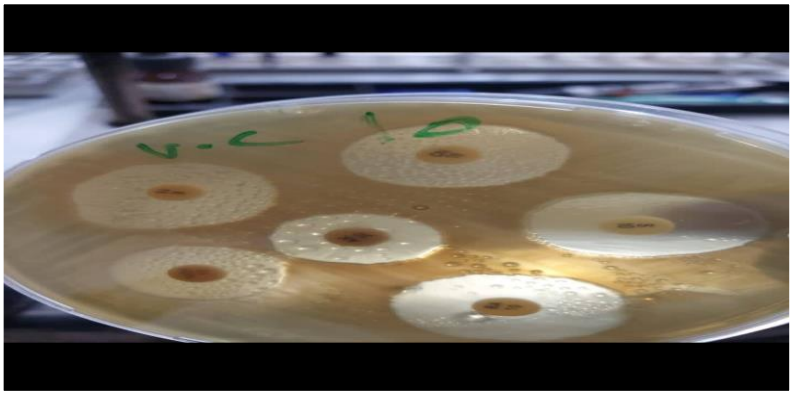

Figure (6): The effect of antibiotic and ascorbic acid(22.2 mg).

In this study, Ascorbic acid has been found to enhance the diameter zone of chloramphenicol in 11 out of 12 samples that turn into sensitive against *P. aeruginosa,* followed by ampicillin, bacterin, and tetracycline with ten samples. While, streptomycin and Trimethoprim-sulfamethoxazole, had a synergistic effect on 9 isolates. Also, the increases of zone diameters appeared with using Gentamicin and Erythromycin in 7 out of 12 samples and in 6 samples by using Amikacin and Ciprofloxacin. In comparison to Norfloxacin alone and tobramycin alone, the average zone of inhibition diameters was reduced in the Norfloxacin (9/12) and tobramycin (8/12) ascorbic acid combination. Antibiotics combined with ascorbic acid have been shown to have a synergistic effect in vitro, increasing the efficacy of treatment against resistant *P. aeruginosa* bacteria. The present results are compatible with a study done by[27] Karim, (2007), Synergistic effect between aminoglycoside and ascorbic acid was shown against *Pseudomonas aeruginosa* bacteria. Also, Erythromycin and SXT showed a synergistic effect with ascorbic acid toward *Pseudomonas aeruginosa* isolates. This study also agrees with [28], In vitro tests were conducted against 12 multi-resistant *Pseudomonas aeruginosa* isolates using a combination of ascorbic acid (AA) and 12 antibiotics. AA chloramphenicol, streptomycin, and tetracycline all showed synergic action. Any antibiotic was shown to be ineffective, with the exception of Amikacin, which was shown to be antagonistic. the results from the current study were revealed that ascorbic acid somehow increases the antibiotic sensitivity and the increase in the zone of inhibition was observed with chloramphenicol,

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ampicillin, bacterin, tetracycline, streptomycin, Trimethoprim-sulfamethoxazole, Gentamicin, Erythromycin, and Ciprofloxacin [29]. According to studies, ascorbic acid suppresses *P. aeruginosa* growth by interfering with sugar absorption via membrane components such as the phosphotransferase system; this system is sensitive to acerbate and is reversibly inhibited by ascorbic acid.

The synergistic effect of ascorbic acid with antibiotics may be due to ascorbic acid's effect on some metabolic activity associated with protein synthesis inside bacterial cells, making the organisms more permeable to antibiotics through its effect on the cell membrane, allowing antibiotics to penetrate the cell more easily and effectively, or it could be due to the effect of (H2O2) produced by the auto-oxidation of ascorbic acid, which causes antibiotics to have a higher potency [30]. It's important to note that ascorbic acid is required in human tissues, and the RDA for adult nonsmoking men and women is "120" mg per day [31]. According to findings from the heart and reproductive research, regular dosages of ascorbic acid (100 or 500 mg/day) can lower the risk of heart attack and boost the pregnancy rate [32].Ascorbic acid is also commonly employed as a food additive and preservative, as well as a significant antioxidant in the pharmaceutical and cosmetic sectors and Increased ascorbic acid levels in the blood may limit the efficiency of antibiotic therapy with chloramphenicol, hence increased AA intake is of particular concern, for example, but it might be useful in combination with other antibiotics like tetracycline. Because ascorbic acid levels are likewise elevated on human tears when vitamin C -1 g/day is supplemented, it might interact with both systemic and on-topic antibiotics, such as those used to treat eye infections [30].

Conclusions

We can conclude that, The sensitivity assay showed a high synergistic activity of ascorbic acid when mixed with antibiotics to increase the potency of the antibiotics against *Pseudomonas* bacteria, especially the antibiotic chloramphenicol. Ascorbic acid has an antagonistic effect for some antibiotics such as norfloxacin and tobramycin, as the area of inhibition for these antibiotics decreased when the vitamin was present.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the corresponding author (Khairi).

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Competing Interests

The authors declare no conflict of interest regarding thisreport.

Author Contributions

(Khairi) made the design of the study, the writing of thearticle, the treatment and controls, and the revision of themanuscript. IS provided data collection, article writingrevision of the article draft, submission of the article andentry of subsequent revisions into the system.

References

[1]. Milivojevic, D.; Neven, S.; Strahinja, M.; Aleksandar, P.; Ivana, M.; Branka, V.; Lidija, S. and Jasmina, N. R. (2018). Biofilm-forming Ability and infection potential of Pseudomonas aeruginosa Strains isolated from animals and humans. Pathogens and Disease, 76: 1-3.

[2]. Streeter, K.; Katouli, M. (2016). Pseudomonas aeruginosa: A Review of Their Pathogenesis and Prevalence in Clinical Settings and the Environment. Infect Epidemiol Med., 2:25-32.

[3]. Haenni M; Hocquet, D.; Ponsin, C. (2015). Population Structure and Antimicrobial Susceptibility of Pseudomonas aeruginosa from Animal Infections in France. BMC Vet Res., 11.

[4]. 21Gul, A.A.; Ali, L.; Rahim, E. and Ahmed, S. (2007). Chronic suppurative otitis media; frequency of *Pseudomonas Aeruginosa* in patients and its sensitivity to various antibiotics. *Professional Med J.* 14(3): 411-5.

[5]. 34.Moshi, N. H.; Minija, B. M.; Ole-Lengine, L. and Mwakagile, D. S. (2000). Bacteriology of chronic otitis media in Dares Salaam, Tanzania. *East Afr Med J*. 77(1): 20-2.

[6]. 22. Hancock, R. E. and Speert, D. P. (2000). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug. Resist. Updat.*,3, 247-255.

[7]. 11.Brown, D.J. (1996). Herbal Prescriptions for Better Health. Rocklin, CA: Prima Publishing. Pp: 213-4.

[8]. 13.Cathcart, R. F. (1991), A unique function for ascorbate. *Med. Hypoth*., 35, 32-37.

[9]. Murray, P. R.; Baron, E. J.; Jorgensen, J. H.; Landry, M. L.; Pfaller, M. A. (2007). Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Manual of clinical microbiology. 9th ed. Washington, DC: American Society for Microbiology, 1152-72.

[10]. Clinical and Laboratory Standards Institute (CLSI). (2020). Performance Standards for Antimicrobial Susceptability Testing. 22nd Informational Supplement. CLSI document M100-S22, Wayne, P. A.: Clinical and Laboratory Standards Institute. 32 (3).

[11]. Grewal, U. S., Bakshi, R., Walia, G. and Shah, P. R. (2018). Antibiotic Susceptibility Profiles of Non-fermenting Gram-negative Bacilli at a Tertiary Care Hospital in Patiala, India. *Niger Postgrad Med J* .24:121- 125.

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http://www.veterinaria.org
Article Received: 25 March 2022; Revised: 15 April 2022; Accepted: 17 May 2022; Publication: 02 July 2022

[12]. Shaheen, M.; Tantary, H. A. and Nabi, S. U. (2016). A Treatise on Bovine Mastitis: Disease and Disease Economics, Etiological Basis, Risk Factors, Impact on Human Health, Therapeutic Management, Prevention and Control Strategy. J Adv Dairy Res., 4: 1.

[13]. Azhar, A. N. (2017). Molecular Detection of virulence factor genes in Pseudomonas aeruginosa isolated from human and animals in Diwaniya province. KufaJournalFor Veterinary Medical Sciences, 8: 218- 226.

[14]. Abdul-Kareem, K. and AL-Hassab, H. (2014). Detection of some virulence factors of pseudomonas aeruginosa isolated from raw milk and soft cheese. vet. medicine collage - Baghdad University. M. V. Sc. Thesis. P: 98.

[15]. Chambers, H.F. (2017). Chemotherapeutic drugs. In: Basic and Clinical Pharmacology. Katzung, B. G. (8th Ed.). Lange Medical Books/McGraw-Hill. USA.

[16]. Baron, E.J., Finegold, S. M. and Peerson, L. R. (1994). "Baily and scott's Diagnostic microbiology" (9th Ed.). Mosby Company. Missouri. P. 389-395.

[17]. Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A. and Wright, G.D. (2012). Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One*, 7(4): e34953.

[18]. Merlin, T. L.; Corvo, D. L.; Gill, J. H. and Griffith, J. K. (2018). Notes: Enhanced gentamycin killing of E.coli by tel gene expression. J. Antimicrob. *Agents. Chemother*., 33:230-232.

[19]. Damron, F. H. and Goldberg, J. B. (2013).Proteolytic regulation of alginate overproduction in *Pseudomonas aeruginosa*. *Mol. Microbiol.,* 84(4):595-607.

[20]. Lysczak. J. B.; Cannon. C. L.; Pier. G. B. (2002). Establishment of *Pseudomonas aeruginosa*infection: lessons from a versatile opportunist. *Microbes Infect*., 2:1051-60.

[21]. Campos, M.A.; Arias, A.; Rodriguez, C.; Dorta, A.; Betancor, L.; Lopez-Aguado, D. and Sierra, A. (1995). Etiology and therapy of chronic suppurative otitis media. *Chemother. J.* 7(5): 427-31.

[22]. Aslam, M.A.; Ahmed, Z. and Azim, R. (2005). Microbiology and drug sensitivity patterns of chronic suppurative otitis media. *Coll Physicians Surg Park J.* 15(6):378-9.

[23]. 35.Moshi, N. H.; Minija, B. M.; Ole-Lengine, L. and Mwakagile, D. S. (2000). Bacteriology of chronic otitis media in Dares Salaam, Tanzania. *East Afr Med J*. 77(1): 20-2.

[24]. Poorey, V.K. and Layer, A. (2002). Study of bacterial flora in CSOM and its clinical significance. *Indian Journal of Otolayngology and Head and Neck Surgery*.54(2):91-5.

[25]. AL-Khazali, K. A. (2009). Resistance of *Pseudomonas aeruginosa* and Staphylococcus aureusisolated from Burns and Wounds infections to Antibiotics and some Disinfectants. M.SC. Thesis, college of science, AL-Mustansriyia University.

[26]. AL-Mayyahi, A.W. J. (2018). Detection of (exoT,exoY,exoSandexoU) Genes in *Pseudomonas aeruginosa*Isolatefrom Different Clinical Sources. M.SC. Thesis, College of Science, University of Baghdad.

[27]. Karim , ArwaHammodi (2007). Effect of Tris-EDTA and ascorbate in increasing antibiotic activity against bacteria isolated from Otitis Media . Master Thesis Submitted to the College of Science of Al-Nahrain University.

[28]. Luciana. C, Edmar. C. A. and Andréa M, A, (2015). Synergic interaction between ascorbic acid and antibiotics against *Pseudomonas aeruginosaBraz. arch. biol. technol*. 48(3) .

[29]. Loewen PC, Richter H. E. (2009). Inhibition of sugar uptake by ascorbic acid in *Pseudomonas aeruginosa*. *Arch BiochemBiophys.,* 226: 657-665.

[30]. Kramarenko, G.G.; Hummel, S.G.; Martin, S.M. and Buettner, G.R. (2007). Ascorbate Reacts with Singlet Oxygen to Produce Hydrogen Peroxide. *PhotochemPhotobiol*. 82(6): 1634-1637.

[31]. Graham, G.; Danuta, S.; Salva, E.; Chris, B.; Erica, W.; Neely, S.; Kensuke, M.; Paul, K.; Zbytnuik, D.; Ling, M.; Xiaobin, X.; Donald, E and Christopher, H. (2014). Different Domain of *Pseudomonas aeruginosa*exoenzyme S active distinct TLRs. *J. Immunol*. 173(3):2031-2040.

[32]. Tabak, M.; Armon, R.; Rosenblat, G.; Stermer, E. and Neeman, I. (2003), Diverse effects of ascorbic acid and palmitoylascorbate on Helicobacter pylori survival and growth. *FEMS MicrobiolLett*., 224, 247-253.