

Pathogenicity, Plasmid Profile and Effectiveness of Some Herbal Extracts on *Pseudomonas fluorescens* Isolated from *Oreochromis niloticus* Fish

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Abstract: *Pseudomonas fluorescens* is one of serious fish diseases in Egypt responsible for sever economic losses. Four *P. fluorescens* isolates were previously isolated from outbreaks in Al-Abbassa and Al-Fayoum farms were used in this study. The plasmid profile of all isolates showed the pattern of two bands. Disc diffusion method was used to test the potency of some natural plant extracts in controlling *P. fluorescens* infections. The most effective methanolic extracts of plants and substances were Propolis, Thyme (*Thymus vulgaris*) whole plant, Juniper (*Juniperus communis*) fruits, Myrrh (*Commiphora molmol*) oleoresin, Aloe (*Aloe vera* L.), dried gel, Tamarind (*Tamarindus indica*) paste and Tea (*Camellia sinensis*) leaves. Other extracts didn't show any inhibition zone and presumptively not effective.

Key words: *Pseudomonas fluorescens* • *Oreochromis niloticus* • Plasmids • Natural Plant Extracts

INTRODUCTION

Increasing the demand for protein, Nile Tilapia (*Oreochromis niloticus*) has attained a great economic importance in Egypt. Infectious diseases of cultured fish are the most impediments on the expansion and development of aquaculture. Bacterial fish pathogens under these stress factors of intensive culture would prevail causing severe economic losses with 80% of mortalities and intricate health hazard both in Egypt and worldwide [1, 2].

P. fluorescens is gram-negative, motile, rod shape and highly heterogeneous species of γ -Proteobacteria known for their versatile metabolism and for their ability to colonize different ecological niches that inhabit soil, plants and water surfaces. The optimum growth temperature is between 25 to 30°C. It has been reported to cause disease in a wide range of fishes. *Pseudomonas* infection in fish leads to development of the so called Red skin disease, which occurs throughout the year especially at low temperatures. The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites and exophthalmia [2, 3].

Some strains of *P. fluorescens* found to be opportunistic human pathogens. MFN1032 is a clinical strain, belonging to *P. fluorescens* biovar I which was isolated from a patient with a lung infection and is able to

grow at 37°C [4]. Plasmids are common in many species of *Pseudomonas*. Conjugative plasmids are an essential factor in the evolution of these bacteria. Plasmids of *Pseudomonas* spp. are carrying genes for virulence, antibiotics resistance, phytotoxin and phytohormone biosynthesis, UV tolerance and bioremediation of polluted environments [5-7].

Natural plant products present a good alternative to chemotherapeutics and antibiotics and other banned drugs being safer for the reared organism and humans. Moreover, these substances also possess other valuable properties; they are nontoxic, biodegradable and biocompatible, inexpensive, easy to get and to produce locally in our country.

MATERIALS AND METHODS

Samples and Biochemical Identification: A total of four strains of *P. fluorescens* were isolated from moribund *O. niloticus* from Al-Abbassa governmental fish farms and Al-Fayoum private fish farms, Egypt. The strains were isolated from the kidney of individual diseased fish and cultivated on *Pseudomonas* selective agar base (LabM, UK) supplemented with CFC supplement. The suspected *Pseudomonas* isolates were identified by oxidase, catalase. The isolates were then biochemically identified using API20NE strips (BioMerieux, France) according to manufacturer's instructions.

Plasmid DNA Isolation: Plasmid miniprep kit (Thermo Scientific, USA) was used, as recommended by the manufacturer, to extract <50-kb plasmids from the isolates. The plasmid preparations were separated by regular gel electrophoresis (0.8%) at 70 V with TAE electrode buffer. The gel was stained with ethidium bromide (1 mg/L) for 15 min and was visualized and photographed the bands under Gel Documentation system (BioRad).

Experimental Infection: Apparently healthy *O. niloticus* fish (10 fish per tank), weighting 50 ± 5 gram were selected for determination of the pathogenicity of the bacterial isolates. Bacterial cultures of each *Pseudomonas* strain were adjusted to a predetermined dose of 3×10^7 CFU. Groups of 10 fish were intra-peritoneally injected with 0.1ml of *P. fluorescens*. Fish were observed daily for 7 days for lesions and mortality.

Histopathology: For histological investigation, organs such as hepatopancreas, posterior kidney, spleen and gills were dissected out from infected fish and control fish. The organs were fixed in Davidson's fixative. After 24 hours they were transferred to 70% ethanol for further ordinary histopathological processing [8].

Potency of Herbal Extracts Against *P. fluorescens*: Methanolic extracts of 16 traditional medicinal plants and substances from Linden (*Tilia platyphyllys*) leaves, Chinese Dar (*Cinnamomum cassia*) park, Salvia (*Salvia miltiorrhiza*) leaves, Meswak (*Salvadora persica*) roots, Propolis, Oregano (*Origanum vulgare*) whole plant, Thyme (*Thymus vulgaris*) whole plant, Juniper (*Juniperus communis*) fruits, Myrrh (*Commiphora molmol*) oleoresin, Cinnamon (*Cinnamomum verum*) park, Cinchona (*Cinchona officinalis*) park, Basil (*Ocimum basilicum* L.) whole plant, Bees wax (*Cera alba*), Aloe (*Aloe vera* L.) dried gel, Tamarind (*Tamarindus indica*) paste and Tea (*Camellia sinensis*) leaves were used in this study. A 20 μ l of each undiluted herbal extract was added to the sterile filter paper disc created from 3 layers of Whatman #1 filter paper and left to dry under sterile condition at room temperature. The prepared discs were then used. One disc without antibiotic was used as a negative control. The potency of antimicrobial agents was tested by the disc diffusion test. Briefly, an overnight culture in IST broth was diluted in sterile 0.85% saline; spread the inoculum over the entire surface of the Muller Hinton plate by three dimensional way. A selective of discs has been applied to the surface of the agar within 15 minutes of inoculation. Each disc was pressed down firmly on to

the surface of the agar plate using sterile forceps. The plates were incubated at 30°C for 24 h for development of inhibition zone around the discs. The test was carried out in triplicates and the diameter of the zone was measured and the results were tabulated as positive or negative antimicrobial activity [9].

RESULTS

Clinical Signs: To verify the pathogenicity of *Pseudomonas* isolates, selected strain from each species was injected into a group of fish. *P. fluorescens* strains showed variable mortality rates, the strain FP2 caused death of all fishes within 24 hours, followed by strain FP6 which caused (80%) death of fishes. Al-Abbassa strains were highly virulent and caused death for 80% of fishes within 72 hrs.

The challenged fish exhibited signs of dark pigmentation, easily detached scales, petechial hemorrhage on different parts of the body surface and ulceration, especially at dorsum part and at the base of fins with eroded fins. Results of re-isolation of bacteria and its identification revealed the same characters of the injected bacteria.

Plasmid DNA Isolation: In this study, the results of plasmids isolation from 4 *P. fluorescens* showed that, all isolates had the pattern of 2 bands representative for two plasmids of different size. The plasmids preparation was run against 1 kbp ladder and the size of plasmids was; one plasmid of about ~3.5kb and other plasmid band was above the ladder of about >10kb (Fig 1).

Histopathology: Circulatory, degenerative, proliferative and necrotic changes were evident. Moreover, some bacterial agents were detected in histological sections.

Haemorrhages were noticed extensively in haemopoietic tissues. Moreover, Vascular and sinusoidal congestions diffuse vacuolar degeneration and discrete necrotic changes were commonly evident in hepatocytes as well as pancreatic acinar cells (Fig. 2a).

Congestion of renal and splenic blood vessels also detected. Moreover, diffuse vacuolation of tubular epithelial cells and necrotic areas were frequently noticed in glomerulo-tubular and interstitial tissues. These necrotic areas were replaced by empty spaces and tissue debris. Furthermore, multifocal depletion of renal and splenic haemopoietic elements was eminent concurrently with hyper-activation of melanomacrophage centers and multifocal infiltration of mononuclear cells (Fig. 2b, c).

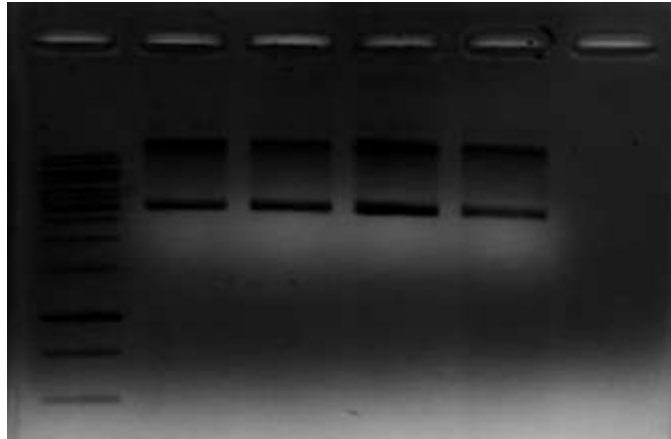


Fig. 1: Agarose gel electrophoresis of plasmid profile of some *P. fluorescens* isolates. Lane 1: kb molecular DNA marker. Lane 2 and 3: two *P. fluorescens* strains from Al-Fayoum governorate bearing two plasmids. Lane 4 and 5: two *P. fluorescens* strains from Al-Abbasa governmental farms bearing the same plasmids.

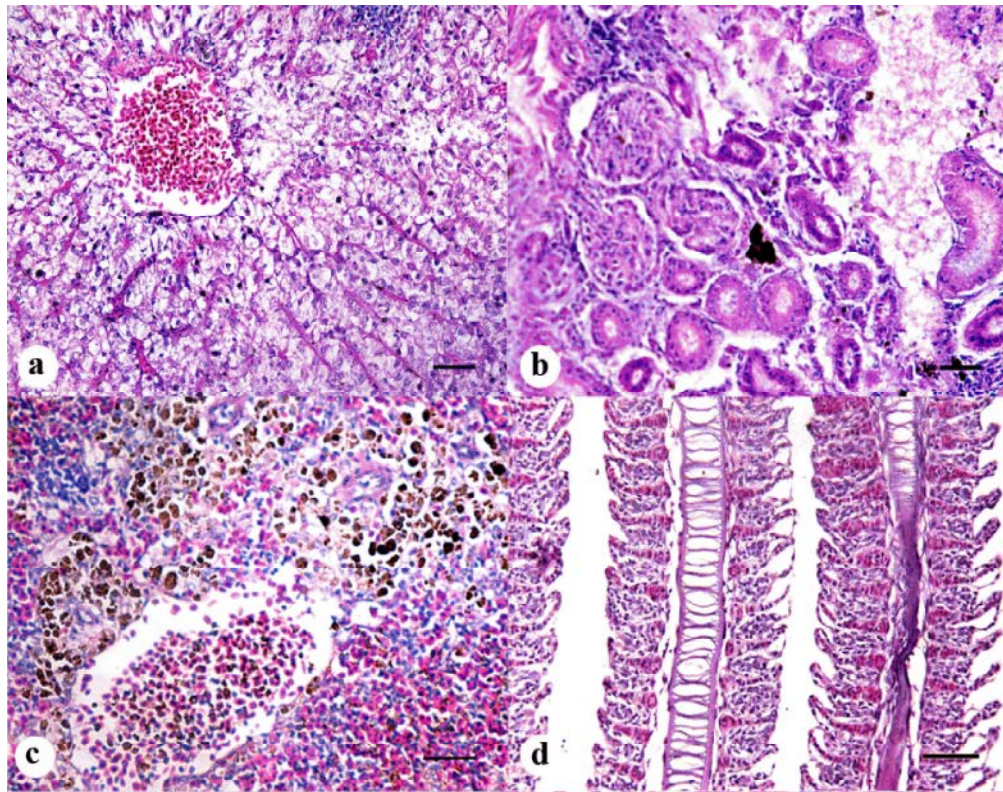


Fig. 2: (a) Hepatopancreas of *O. niloticus* experimentally infected with *P. fluorescens* after 48 hours showing congestion of main blood vessels and sinusoidal spaces with diffuse vacuolar degeneration and hepatocytic necrosis. (b) Posterior kidney of *O. niloticus* experimentally infected with *P. fluorescens* after 48 hours showing multifocal necrosis of the interstitial tissue with discrete tubular necrosis leaving cyst like spaces. (c) Spleen of *O. niloticus* experimentally infected with *P. fluorescens* after 48 hours showing congestion and multifocal necrosis with discrete depletion of white pulp with activation of melanomacrophage centers. (d) Gills of *O. niloticus* experimentally infected with *P. fluorescens* after 48 hours showing separation in-between the epithelial cell lining of the secondary gill lamellae and the underlying capillary bed with hyperplasia of the epithelial lining at the base of the secondary gill lamellae. (Bar = 50µm).

Table 1: Effects of some herbal extracts against three *Pseudomonas fluorescens* strains by using disc diffusion method.

Plant extract or substance	Concentration	<i>P. fluorescens</i> strains			Plant extract or substance	Concentration	<i>P. fluorescens</i> strains		
		FP2	FP6	BP1			FP2	FP6	BP1
Linden (<i>Tilia platyphyllos</i>) leaves	25%	-	-	-	Myrrh (<i>Commiphora molmol</i>) oleoresin	50%	+	+	+
Chinese Dar (<i>Cinnamomum cassia</i>) park	10%	-	-	-	Cinnamon (<i>Cinnamomum verum</i>) park	25%	-	-	-
Salvia (<i>Salvia miltiorrhiza</i>) leaves	7%	-	-	-	Cinchona (<i>Cinchona officinalis</i>) park	20%	-	-	-
Meswak (<i>Salvadora persica</i>) roots	10%	-	-	-	Basil (<i>Ocimum basilicum</i>) whole plant	25%	+	+	+
Propolis	20%	+	+	+	Bees wax (<i>Cera alba</i>)	10%	-	-	-
Thyme (<i>Thymus vulgaris</i>) whole plant	10%	-	-	-	Aloe (<i>Aloe vera</i>) dried gel	10%	+	+	+
Oregano (<i>Origanum vulgare</i>) whole plant	10%	-	-	-	Tamarind (<i>Tamarindus indica</i>) paste	10%	+	+	+
Juniper (<i>Juniperus communis</i>) fruits	50%	+	+	-	Tea (<i>Camellia sinensis</i>) leaves	10%	+	+	+

The gills showed congestion of lamellar blood vessels, diffuse and multifocal hyperplasia of epithelial lining of the secondary lamellae (Fig. 2d).

Potency of Herbal Extracts Against *P. fluorescens*: Disc diffusion method was followed to evaluate the antibacterial activity against three *P. fluorescens* isolates. No zone of inhibition was seen in the control disc without antibiotics. As shown in Table 1, some herbal extracts had variable zone of inhibition and were found to be effective in controlling the *Pseudomonas* infection in Tilapia fish. These extracts and substances were; Propolis, Thyme (*Thymus vulgaris*) whole plant, Juniper (*Juniperus communis*) fruits, Myrrh (*Commiphora molmol*) oleoresin, Aloe (*Aloe vera* L.) dried gel, Tamarind (*Tamarindus indica*) paste and Tea (*Camellia sinensis*) leaves. Other extracts didn't show any inhibition zone and presumptively not effective. These findings indicated that these extracts can be developed as preferred natural agents for the treatment of *P. fluorescens* isolates.

DISCUSSION

P. fluorescens is an aquaculture pathogen that has been reported to cause disease in a wide range of fish species, including Indian major carps, black carp, common carp and Japanese flounder. *P. fluorescens* is considered to be one of the primary causes of pseudomonas septicemia, tail and fin rot in fish, with all over the year with maximum prevalence in summer [3, 10]. *P. fluorescens* is one of the most pathogenic bacteria affecting fish farms in Egypt. *P. fluorescens* Biovar I (15 isolates), *P. fluorescens* Biovar II (17 isolates), *P. fluorescens* Biovar III (13 isolates) were isolated from 480 fish in Egypt [11].

The result of plasmid isolation showed that all isolates either from Al-Abbassa or from Al-Fayoum have the same plasmid profile bearing two plasmid of about ~3.5kb and other plasmid band was >10kb. In accordance with this result, a variety of plasmids have been isolated from *Pseudomonas* species such as R-factor plasmids,

which inactivate certain antibiotics and S-factor plasmids capable of initiating chromosomal gene transfer. Plasmids in *Pseudomonas* species are also known to code for heavy metal resistance and degradation of organic compounds [12]. Analysis of 10 strains, only one strain of *P. fluorescens* A506, harbored a conjugative plasmid of 57-kb [7]. Also, plasmid DNA was isolated from *P. aeruginosa* and designated as pBC15. The size of the plasmid DNA was approximately 23 kb. The *Pseudomonas aeruginosa* isolate exhibited resistance to heavy metals such as cadmium, chromium, nickel and lead [13]. Members of the *Enterobacteriaceae* family and the genus *Pseudomonas* carry plasmids belonging to more than 30 Incompatibility (Inc) groups especially (IncP, W, N and Q) which can transfer between and maintain themselves in both enteric bacteria and strains of *Pseudomonas* [14].

Regarding histopathological lesions noticed in infected fishes, circulatory, degenerative, proliferative and necrotic changes were evident. Destruction of the critical components of the circulatory and immune system by toxic bacterial extracellular products is thought to be the corner stone behind these pathological alterations [15, 16]. Among these ECPs, proteases and haemolysins are at the forefront, contributing significantly to the ruinous nature of these diseases [17, 18].

Plant essential oils have been used for hundreds of years as natural medicines to combat bacteria, fungi and viruses. Several essential oils confer antimicrobial activity by damaging the cell wall and membrane, leading to cell lysis, leakage of cell contents and inhibition of proton motive force [19, 20]. Herbs or herbal products also have a role in aquaculture at present time, because it is natural and biodegradable and has antimicrobial activity. Several medicinal plants have shown activity against many important pathogenic bacteria of fish.

Similar to our results Kavanaugh and Ribbeck [20] found that cassia, clove (*Syzygium aromaticum*), Peru balsam (*Myroxylon balsamum*), red thyme (*Thymus vulgaris*) and tea tree (*Melaleuca alternifolia*) oils were effective in killing *P. aeruginosa*. The essential oil

composition of *Thymus vulgaris* include mainly thymol and carvacrol [21]. In addition, five herbs *Allium sativum*, *Calotropis gigantea*, *Momordica charantia*, *Polygonum hydropiper*, *Psidium guajava* found to be highly effective against three high virulent aquaculture bacteria *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda* [22]. Similarly, aqueous extracts of common fruits, herbs and spices (raspberry, blueberry, blackberry, cranberry, grape, oregano, thyme, basil, kale, ginger, turmeric) significantly inhibited quorum sensing by the inhibition of swarming motility in the case of pathogenic *P. aeruginosa* and *E. coli* [23]. Also, propolis may be a useful adjunctive agent for traditional antibiotic therapy for *P. aeruginosa* keratitis in rabbits [24].

CONCLUSION

The result of plasmid isolation of *P. fluorescens* showed that all isolates either from Al-Abbassa or from Al-Fayoum have the same plasmid profile bearing two plasmids and could play a role in pathogenesis and resistance of this pathogen. Due to the increased prevalence of antibiotic resistance, using of antibiotics in aquaculture needs to be reduced and replaced with alternative effective medicinal plants against the bacterial diseases. These preliminary findings provided that some extracts were found to be effective in treatment of *P. fluorescens* and can be used for treating bacterial infection in fish farms.

REFERENCES

1. Plumb, J.A., 1999. Overview of warmwater fish diseases. *Journal of Applied Aquaculture*, 9(2): 1-10.
2. Austin, B. and D.A. Austin, 2007. *Bacterial fish pathogens: disease of farmed and wild fish*, Springer Science & Business Media, pp: 552.
3. Swain, P., A. Behura, S. Dash and S. Nayak, 2007. Serum antibody response of Indian major carp, *Labeo rohita* to three species of pathogenic bacteria; *Aeromonas hydrophila*, *Edwardsiella tarda* and *Pseudomonas fluorescens*. *Veterinary immunology and Immunopathology*, 117(1): 137-141.
4. Chapalain, A., G. Rossignol, O. Lesouhaitier, A. Merieau, C. Gruffaz, J. Guerillon, J.M. Meyer, N. Orange and M. Feuilloley, 2007. Comparative study of 7 fluorescent pseudomonad clinical isolates. *Canadian Journal of Microbiology*, 54(1): 19-27.
5. Frost, L.S., R. Leplae, A.O. Summers and A. Toussaint, 2005. Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology*, 3(9): 722-732.
6. Strateva, T. and D. Yordanov, 2009. *Pseudomonas aeruginosa*—a phenomenon of bacterial resistance. *Journal of Medical Microbiology*, 58(9): 1133-1148.
7. Stockwell, V.O., E.W. Davis, A. Carey, B.T. Shaffer, D.V. Mavrodi, K.A. Hassan, K. Hockett, L.S. Thomashow, I.T. Paulsen and J.E. Loper, 2013. pA506, a conjugative plasmid of the plant epiphyte *Pseudomonas fluorescens* A506. *Applied and Environmental Microbiology*, 79(17): 5272-5282.
8. Roberts, R.J., 2012. *Fish pathology*, John Wiley & Sons., pp: 581.
9. Wikler, M.A., 2006. Performance standards for antimicrobial susceptibility testing: Sixteenth informational supplement, Clinical and Laboratory Standards Institute.
10. Zhang, W.W., Hu, Y.H., Wang, H.L. and L. Sun, 2009. Identification and characterization of a virulence-associated protease from a pathogenic *Pseudomonas fluorescens* strain. *Veterinary Microbiology*, 139(1): 183-188.
11. Eissa, N., E.A. El-Ghiet, A. Shaheen and A. Abbass, 2010. Characterization of *pseudomonas* species isolated from Tilapia *Oreochromis niloticus* in Qaroun and Wadi-El-Rayan lakes, Egypt. *Global Veterinaria Journal*, 5: 116-121.
12. Palchadhuri, S. and A. Chakrabarty, 1976. Isolation of plasmid deoxyribonucleic acid from *Pseudomonas putida*. *Journal of Bacteriology*, 126(1): 410-416.
13. Raja, C.E. and G. Selvam, 2009. Plasmid profile and curing analysis of *Pseudomonas aeruginosa* as metal resistant. *International Journal of Environmental Science & Technology*, 6(2): 259-266.
14. Popowska, M. and A. Krawczyk-Balska, 2013. Broad-host-range IncP-1 plasmids and their resistance potential. *Front. Microbiol.*, 4(44): 1-8.
15. Kimura, H. and R. Kusuda, 1979. Studies on the Pathogenesis of Streptococcal Infection in Cultured Yellowtails *Seriola* spp. - Effect of the Cell Free Culture on Experimental Streptococcal Infection. *Journal of Fish Diseases*, 2(6): 501-510.
16. Woo, P.T., 2011. *Fish Diseases and Disorders: Volume 3: Viral, Bacterial and Fungal Infections*, Cabi.
17. Pazos, F., 1997. *Flexibacter maritimus*: estudio fenotípico, inmunológico y molecular. Universidad Santiago de Compostela Thesis. Doctoral.

18. Li, J., L.R. Zhou and N.Y.S. Woo, 2003. Invasion route and pathogenic mechanisms of *Vibrio alginolyticus* to silver sea bream *Sparus sarba*. Journal of Aquatic Animal Health, 15(4): 302-313.
19. Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology, 94(3): 223-253.
20. Kavanaugh, N.L. and K. Ribbeck, 2012. Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. Applied and Environmental Microbiology, 78(11): 4057-4061.
21. Thompson, J.D., J.C. Chalchat, A. Michet, Y.B. Linhart and B. Ehlers, 2003. Qualitative and quantitative variation in monoterpene co-occurrence and composition in the essential oil of *Thymus vulgaris* chemotypes. Journal of Chemical Ecology, 29(4): 859-880.
22. Muniruzzaman, M. and M. Chowdhury, 2013. Evaluation of medicinal plants through fish feed against bacterial fish disease. Progressive Agriculture, 19(2): 151-159.
23. Vattam, D., K. Mihalik, S. Crixell and R. McLean, 2007. Dietary phytochemicals as quorum sensing inhibitors. Fitoterapia, 78(4): 302-310.
24. Onlen, Y., C. Tamer, H. Oksuz, N. Duran, M.E. Altug and S. Yakan, 2007. Comparative trial of different anti-bacterial combinations with propolis and ciprofloxacin on *Pseudomonas keratitis* in rabbits. Microbiological Research, 162(1): 62-68.