Isolation and identification of *pseudomonas aeruginosa* from infected sheep and detection of phosolipase C (lecithinase)

Ahmed N. Mahmood and Assma H. Aljobori
Department of Microbiology, College of Veterinary Medicine, Baghdad University, Iraq.
E-mail: dr.ahmedneama@gmail.com
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Summary

The current study dealt with the isolation and identification of 19 isolates (14.07%) for the period (October 2013 to January 2014) from different samples (135 samples) number of isolates belonging to *Pseudomonas aeruginosa* were 8 isolated from urine samples, their percentage were 33.33 % and 5 isolates from nasal swabs, (11.11%) and 4 isolated from milk samples (9.30%), 2 isolated from wound swabs (24%) and isolates from eye infection (9.09%). These isolates were identified by morphological examination and biochemical tests and API-20 NE system. The second part was the study of the antibiotic susceptibility that was carried out on 19 isolates of *P. aeruginosa* for 12 types of antibiotic. The results showed that isolates were resistant to 9 out of 12 antibiotics with percentage (Penicillin 100%, nalidixic acid100%, pipracillin 100%, erythromycin 68,4%, Chloramphenicol 63%, 78%, trimthoprim 73%, ceftacizidem 89%, cefitriaxon 84% and cefotaxime 89%) on the other hand most of isolates were sensitive to (epipenime, azythromycin, and polymixin). Detection of phospholipase C (Lecithinase) by agar well diffusion method on different selective media as (egg yolk agar, briliant green crystal violates lecithnase agar BCL), 12 isolates out of 19 showed a positive reaction on these media. The result depended on measurement the diameter of opacity zone produced and the blush green zone on the BCL media, the diameter of zone ranged from 8mm to 32mm. both media (EYA and BCL media is the best media for detection of phospholipase by *P. aeruginosa*.

Keyworks: *Pseudomonas aeruginosa*, Sheep, phospholipase C.

Introduction

A variety of diseases of humans and animals are caused by *Pseudomonas aeruginosa*. *P. aeruginosa* is an opportunistic bacterial pathogen that poses a lethal threat to hosts with open injuries and burn wounds, immuno-compromised hosts and above all (1), *Pseudomonas aeruginosa* is a bacterium responsible for severe infections, they typically infects the pulmonary tract, burns, wounds, and also causes other blood infections, pneumonia, septic shock, urinary tract infection, gastrointestinal infection, skin and soft tissue infections(2). *P. aeruginosa* is a common cause of nosocomial infections. Lung infections with this pathogen are associated with high mortality rates in hosts. Also it can cause mastitis in sheep which characterized by swelling of udder, abscesses formation and necrosis (3). *P. aeruginosa* cause severe, progressive, necrotic dermatitis affect wooled area and wool free area (4). *P. aeruginosa* produces various virulence factors. The ability of *P. aeruginosa* to invade tissue depends upon extracellular enzymes and toxins that break down physical barriers and otherwise contribute to bacterial invasion (5) Phospholipase C (lecithinase), an exoenzyme of *P. aeruginosa*, has been identified as a critical component in the pathogenesis of *p. aeruginosa* infection. Among the numerous extracellular products produced by *P. aeruginosa* which may contribute to it's pathogenesis, including toxins, proteases, and exopolysaccharides, there are two hemolysins (6). One of these hemolysins is a heat-stable glycolipid, the other hemolysin is a heat-labile phospholipase C (PLC) which catalyzes the hydrolysis of phosphatidylcholine (7). The phospholipases are a complex and crucially important group of enzymes that hydrolyze phospholipids (PLs) releasing a variety of products, like for example free fatty acids (FFAs), lyso-phospholipids, di-acylglycerols (DGs), choline phosphate and phosphatidates, depending on the site of hydrolysis, they play crucial roles in many biochemical processes related to among others digestion and inflammation. A lot of interest was given to phospholipases from a pharmaceutical

perspective (8). The PLC-H with a mol. wt of 77kDa haemolyses human and sheep erythrocytes and degrades not only phosphorylcholine but also sphingomyelin, which are key components of eukaryotic cell membranes (9). Phospholipases are important virulence factors in an increasing number of intra- and extracellular bacterial pathogens including *Clostridium perfringens*, *Corynebacterium pseudotuberculosis*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* (10). Phospholipases can be divided into four groups depending on the position of the bond they hydrolyse on the phospholipid substrate: phospholipases A1, A2, C and D. Phospholipases C (lecithinase) appear to be the most important playing a significant role in bacterial pathogenesis (11). This study aims to detect the ability of *P. aeruginosa* isolated from infected sheep to produce phospholipase C.

Materials and Methods

Samples were collected from suspected cases of sheep suffering from different infection of both sexes in abu-griab city. The total 135 samples included in this study were 43 milk samples ,18 urine samples, 45 nasal swabs, 22 eye swabs, 8 wound swab, (Table, 1). All samples were placed on brain heart infusion broth then sent to laboratory within 24 hrs. Then they were cultured on blood agar, Maconkey agar and incubated at 37C˚ for 24 hrs. The pale non lactose fermented with dusty odor colonies were selected. Primary identification of isolates was done by biochemical tests and growing on selective media (12), as well as by microscopical examinationby using Gram´s stain, the second step of identification was performed by using Api 20 NE system.The susceptibility of the bacterial isolates to 12 antimicrobial agents including Penicillin, Pipracillin, Cefixime, Ceftriaxone, Ceftazidium, Erythromycin, Azithromycin, Chloramphenicol, Polymixin; Trimethoprime and Nalidixic acid were determined by the disk diffusion method in accordance with NCCLS guidelines. Briefly, diameter of inhibition zone was measured (mm) and compared with the national committee for clinical laboratory standard (13). Detection of phospholipase **C** (Lecithinase) to determine production of lecithinases from *P. aeruginosa*, the isolates were cultured on agar plates containing the appropriate substrates according to (14). Lecithinase production was determined on egg yolk agar containing 10% egg yolk emulsion. After incubation at 30°C for up to 5 days, plates were observed by presence of brown opaque zones surrounded the colonies (15). All the isolates of *P. aeruginosa* were tested for their ability to produce lecithinase enzyme by agar well diffusion method on egg yolk agar, and BCL agar, the positive result depended on measurement the zone diameter produced (16).

Results and Discussion

Out of 135 different samples used in this study 19 isolates belonging to *Pseudomonas aeruginosa* were detected include 4 (9.30%) isolates from milk, 6 (33.33%) isolates from urine, 5 (11.11%) isolates from nasal swabs, 2 (9.09%) isolates from eye swabs and 2 (24%) isolates from wound (Table, 1). They were identified by biochemical tests and microscopic examination as shown in the (Table, 2). The sensitivity test of 19 isolates to 12 type of antibiotic showed that all isolates were resistance to Penicillin 100%, nalidixic acid 100%, pipracillin erythromycin 68.4%, tetracycline, trimthoprim 73%, ceftacizidem 89%, cefitriaxon 84% and cefataxime 89% and sensitive to epipinem 26%, polymxin 36% and azithromycin 57%, (Table, 3). Lecithinase production was determined on egg yolk agar containing 10% egg yolk emulsion. After incubation at 30°C for up to 5 days, plates were observed for the presence of colonies surrounded by brown opaque zones. All the isolates of *P. aeruginosa* were tested for their ability to produce lecithinase enzyme by agar well diffusion method on egg yolk agar, and BCL agar.to choose the isolate that have a greater ability for production, most lecithinase positive organisms will show activity with egg yolk agar (Fig. 1). Twelve isolate showed a positive reaction on the medium. The result depended on measurement the diameter of zone produced. These isolates were numbered from 1 to 12 and the production of phospholipase varied from one isolate to another as shown in the (Table, 4).

Table, 1: No. of *P. aeruginosa* isolates and there percentage from different infection in sheep.

Clinical Isolates	No. of Samples	No. of *P.aeruginosa* Isolates	% of *P.aeruginosa* Isolates
Milk	42	4	9.30 %
Urine	18	6	33.33 %
Nasal swab	45	5	11.11 %
Eye swab	22	2	9.09 %
Wound	8	2	24 %
Total No.	135	19	14.07 %

Table, 2: Biochemical test of *P. aeruginosa*

Biochemical test	Result
gram´s stain	-
Oxidase	+
Growth on MacConkey agar	+
Catalase	+
Urease	V
Gelatine	+
Pyocyanin production	+
Growth at 42°C	+
Growth at 4°C	-
Citrate utilization test	+
TSI test	K/K, -ve , -ve

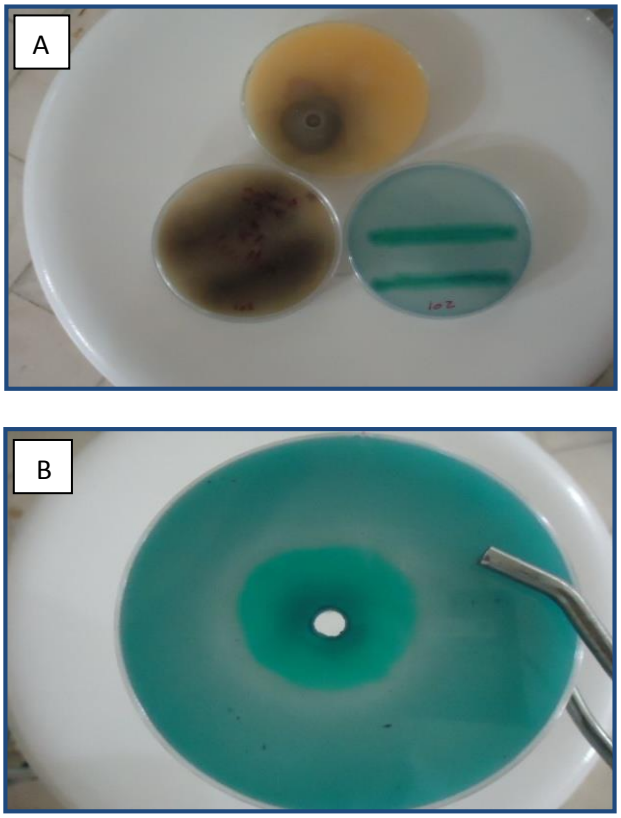
(+) Positive, (–) Negative result; (K/K) Alkaline/ Alkaline

Table, 3: Antibiotic sensitivity test of *P. aeruginosa* isolates in sheep.

Antibiotic	Con. *Micro* g/disc	No of resistant isolates
Nalidixic acid	30	19(100%)
Penicillin	30	19(100%)
Cefotaxime	30	17(89%)
Cephtazidime	30	17(89%)
Ceftriaxone	30	16(84%)
Chloramphenicol	30	12(63%)
Erythromycin	30	13(68.4%)
Trimethoprim	1,5	14(73%)
Polymixin	10	7(36%)
epipenime	10	5(26%)
Pipracillin	10	19(100%)
Ezithromycin	30	11(57%)

Table, 4: Production of phosphlipase C (Diameter) from *Pseudomonas aeruginosa* isolates in sheep.

No. of isolates	D. on EYA (mm)	D. on BCL (mm)
Isolate no 1	29	26
Isolate no 2	22	26
Isolate no 3	11	14
Isolate no 4	22	26
Isolate no 5	8	11
Isolate no 6	10	10
Isolate no 7	18	24
Isolate no 8	10	13
Isolate no 9	12	18
Isolate no10	9	14
Isolate no11	32	26
Isolate no12	16	20



Figure, 1*: P. aeruginosa* isolate on agar well diffusion method: A- *p. aeruginosa* isolate on three different media (EYA, BCL media, and lecithin agar media) *B*- opaque zone on BCL media.

The present study showed that *P. aeruginosa* was one of the main causes of sheep infections. The percentage of isolation of *P. aeruginosa* from infection was (14.07) this result agreed with (17). This study showed that the percentage of isolation ranged from (9.09% to 33.33%) this results agreed with (18) how found the percentage of isolation of California from urinary tract infection 27% and nasal swab 25.5% and from wound 16%. In this study the phospholipase C (lecithinase) activity of *P. aeruginosa* show that 12 isolates out of 19 isolates had lecithinase activity when grow on egg yolk agar (EYA) and brilliant green crystal violet lecithinase agar media (BCL) this result agreed with (16) and with (19) who found 10 from 12 isolates showed their ability to produce PL-C on BCL agar. The growth of these strains is characterized by formation of a grayish-blue opacity surrounded by zone of lysis after 24hr. the observation of PLC in different media showed the suitability of EYA and BCL media for the detection of phospholipase C.

References

1. Donaldson, S. H. and Boucher, R. C. (2003) Update on pathogenesis of cystic fibrosis lung disease, Curr. Opin. Pulm. Med. 9(6): 486–491.
2. Diekema, D. J.; Pfaller, M. A.; Jones, R. N.; Doern, G. V.; Winokur, P. L.; Gales, A. C.; Sader, H. S. and Kugler, K.; et al. (1999). Survey of bloodstream infections due to Gram-negative bacilli: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, and Latin America for the sentry Antimicrobial Surveillance Program, 1997". Clin. Infect. Dis., 29(3): 595– 607.
3. Honhold, N. and Carter, M. E. (1987). *Pseudomonas aeruginosa* mastitis in a sati ewes.Vet. Res., 120:16.
4. Nation, P. N.; Crowe, S. P. and harries,W. N. (1982). Bacterial infection of sheep. Vet. Jur. 76: 122-122.
5. Bouza, E.; Garcia-Garrote, F.; Cercenado, E.; Marin, M. and Diaz, M. S. (1999). *Pseudomonas aeruginosa*: a Survey of Resistance in 136 Hospitals in Spain. Antimicrob. Agents Chemother., (43): 981-982.
6. Vasil, M. L. (1986). Pseudomonas aeruginosa: biology, mechanisms of virulence, epidemiology. J. Pediatr., 108: 800- 805.
7. Esselmann, M. T., and P. V. Liu. (1961). Lecithinase production by gram-negative bacteria. J. Bacteriol., 81:939- 945.
8. Shimizu, T.; Ohto, T. and Kita, Y. (2006) Cytosolic phospholipase A2 Biochemical properties and physiological roles. IUBMB Life. 58: 328– 333.
9. Shortridge, V. D.; Lazdunski, A. and Vasil, M. L. (1992). Osmoprotectants and phosphate regulate expression of phospholipase C in *Pseudomonas aeruginosa.* Mol Microbiol., 6: 863-871.
10. McNamara, P. J.; Bradley, G. A. and Songer, J. G. (1994). Targeted mutagenesis of the phospholipase D gene results in decreased virulence of *Corynebacterium Pseudotuberculosis*. Mol Microbiol., 12: 921–930.
11. Songer, J. G. (2007). Bacterial phospholipases and their role in virulence. Trends Microbiol., 5: 156–161.
12. Atlas, R. M. and Snyder, J. W. (2006). Handbook of Media for Clinical Microbiology. 2 ed. Taylor and Francis group. CRC press. USA.
13. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial disk susceptibility tests. NCCLS documents M 100S15. Wayne, PA, USA: Clinical and Laboratory Standard Institute, (2012).
14. Vanderzant, C. and Splittstoesser, D. F. (1992). Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, D.C., 81: 171- 179.
15. Atlas, R. M.; Parks, L. C. and Brown, A. E. (1995). Laboratory manual of experimental microbiology. Mosby Company. Missouri., 2: 343-355.
16. Esselmann, M. and Liu, P.V. (1961). Lecithinase production by Gram-negative bacteria. J. Bacteriol., 81: 939- 945.
17. Savas, L.; duran, N.; Svasonlen, Y. and Ocak, S. (2005). The pervelance and resistance patterns of *Pseudomonas aeruginosa* in intensive care units university hospital. Turk. J. Med. Sci., 35: 317- 322.
18. Pellegrino, R. L.; Cool, C. S. and Moller, M. G.; Nour, S. A. and Oliveira, M. P. (2002). Occurrence of multidrug resistance *Pseudomonas aeruginosa* clone in different hospital in roide .J.of clini. Microbio., R. J., 40: 2420- 2424.
19. Postupa, R. and Postupova, M. (1988). Lecithinase activity of *Proteus vulgaris.* J. Hyg. Epidemiol. Microbiol., 32(2): 227-231.

عزل وتشخيص بكتريا الزوائف الزنجارية من الأغنام المصابة والتحري عن إنتاج إنزيم الفوسفوليبز(الليسيثنيز)

احمد نعمة محمود و أسماء حمودي الجبوري
فرع الإحياء المجهرية، كلية الطب البيطري، جامعة بغداد، العراق.
E-mail: dr.ahmedneama@gmail.com

الخلاصة

تضمنت الدراسة الحالية عزل وتشخيص 19 عزلة من جرثومة الزوائف الزنجارية (14.07%) من مجموع 135 عينة للفترة (من حزيران 2013 الى يناير 2014) شملت هذه العينات (4 عزلات من مجموع 43 عينة حليب ونسبتها (9.30%) و (6 عزلات من مجموع 18 عينة بول ونسبتها33.33%) و (5عزلات من مجموع 45 مسحة انفية ونسبتها 11.11%) وعزلتان من مجموع 22مسحة عينية ونسبتها 9.09%) و(عزلتان من مجموع 8 مسحات الجروح ونسبتها 24%). تم تشخيص هذه العزلات بالاعتماد على الصفات الزرعية والمجهرية والكيموحيوية ونظام API 20 NE. وتم دراسة حساسية هذه العزلات لعدد من المضادات الحياتية وأظهرت الدراسة مقاومة هذه العزلات ل 9 من المضادات الحياتية من أصل 12 نوع حيث كانت نسبة المقاومة Pipracillin100% و Nalidixic acid 100% و Penicillin 100% و Ceftacizidem 89% و Cefotaxime 89% و Acefitriaxon 84% و Trimthoprim 73% و Erythromycin 68.4% و Chloramphenicol 63% بينما كانت جميع العزلات حساسة للمضادات الحياتية (Epipenime و Azythromycin و Polymixin) وذلك اعتمادا على قياس القطر التثبيطي للمضاد الحيوي. وكذلك تم التحري عن انزيم الفوسفوليبز (الليسيثين) المنتج من هذه العزلات باستخدام طريقة الانتشار بواسطة الاطباق باستخدام عدة انواع من الاوساط الخاصة وهي (Brilliant green crystal violets Lecithinase BCL و Egg yolk agar EYA). 12عزلة من مجموع 19 عزلة اعطت نتيجة موجبة لهذا الاختبار اعتمدت النتيجة على ظهور وقياس قطر العتامة الذي تراوح من 8 مل الى 32مل. كلا الوسطين يعتبر من الاوساط المناسبة للتحري عن انزيم الفوسفوليبز المنتج من قبل بكتريا الزوائف الزنجارية.

الكلمات المفتاحية: الزوائف الزنجارية، الأغنام، الفوسفوليبز ج.