African Journal of Microbiology Research

Volume 10 Number 23 21 June, 2016
ISSN 1996-0808

Academic Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) is provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.me/

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 23 21 June, 2016

ARTICLES

academicJournals

Vol. 10(23), pp. 844-849, 21 June, 2016
DOI: 10.5897/AJMR2016.8042
Article Number: 0D1493759076
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* isolated from clinical and hospital environmental samples in Nasiriyah, Iraq

Jawad R. Al-Zaidi

Southern Technical University, Shatrah Technical Institute, Iraq.

Received 5 April, 2016; Accepted 5 May, 2016

A total of one hundred and forty two swab samples (92 clinical and 50 from hospital environment) were collected for the detection of *Pseudomonas aeruginosa*. Out of the total samples, 29 isolates of *P. aeruginosa* were isolated and recorded an overall prevalence rate of 20.42% (29/242) of which 18 (19.56%) were from wounds and burns swabs of patients, and 11 (22%) were from hospital environment. The highest rate of *P. aeruginosa* (60%) identified from hospital environmental specimens were from door handles followed by ward sinks (57.15%) and the least (10.53%) from patients' beds and table tops. According to gender and age group, the study showed the highest rate of *P. aeruginosa* in the male (55.6%), and in young patients (38.9%) between the ages of 5 and 25 years compared to the elderly; while the lowest rate 27.8% were from those age 45 years and above. Results showed that all isolates from patients and hospital environment were resistant to ticarcillin and ceftazidime (100%). Also, *P. aeruginosa* from patients demonstrated high resistance to cefepime, ofloxacin, gentamycin, tobramycin, ciprofloxacin, lomefloxacin, norfloxacin, levofloxacin and amikacin in the following order respectively :88.8, 77.7, 61.1, 50.0, 44.4, 44.4, 38.8, 38.8 and 33.3%; whereas showed low resistance (16.6 and 11.1%) to each of ticarcillin/clavulanate and meropenem, and only 5.5% to imepenem. Generally, this study pointed that *P. aeruginosa* isolates from hospital environment were more resistant to particular antibiotics than that of clinical isolates. It was also revealed that *P. aeruginosa* have high sensitivity to imepenem, meropenem and ticarcillin /clavulanate and these should be considered in the treatment of this bacterium.

Key words: *Pseudomonas aeruginosa*, antibiotic resistance, clinical samples, surgical wards, hospital environment.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen, it has higher prevalence and mortality rate in hospital environment, especially among patients, particularly those with burns, wounds and cancer and in

*Corresponding author. E-mail: jwdrshd@yahoo.com.

Table 1. Frequency of *P. aeruginosa* isolates among patients and hospital environment specimens, 2012.

Specimen type	Specimens no.	*P. aeruginosa* isolates	
		NO.	%
Clinical samples (Burns and wounds)	92	18	19.56
Environment	50	11	22
Total	142	29	20.42

the critically ill admitted in intensive care unit. *P. aeruginosa* is resistant to several antimicrobial agents and therefore become dominant and important when more susceptible bacteria of the normal flora are suppressed (Brooks et al., 2011; Meenakumari et al., 2011). Contaminated medical devices and the hospital environment have often been suggested as potential sources infection with *P. aeruginosa* and the high mortality associated with these infections is due to a combination of the bacterial resistance to antibiotics and a weak host defense system (Meenakumari et al., 2011; Orsi et al., 1995). Wound and burn infections is a major contributor to nosocomial infections. Furthermore, these infections prolong the burden of the disease by increasing cost of treatment, hospital stay and sometimes may lead to death particularly when complicated with septicemia and tetanus (Sule et al., 2002). The current spread of multi-drug resistant bacteria pathogens such as *P. aeruginosa* has added a new side to the problem of wound and burn infections (Sule and Olusanya, 2000). These strains of *P. aeruginosa* establish themselves in the hospital environment in areas like, door handles, patients' beds, sinks, table tops, toilets and others, thereby spreading from one patient to another: and it has a high rate for developing resistance to most of the antimicrobial agents (Haghi et al., 2010; Falagas et al., 2005). Therefore, this study aimed to detect the antibiotic susceptibility of *P. aeruginosa* isolated from burns and wounds of patients and hospital environmental samples from Al Hussein Teaching Hospital in Nasiriyah, Iraq.

MATERIALS AND METHODS

Isolation and identification of bacteria

Clinical swabs from wounds and burns were collected from hospitalized patients in burns and wounds wards and from items in the hospital environment, and subsequently examined from May to August, 2012. One hundred and forty two samples (92 from patients and 50 from items in hospital environment: patients' beds, door handles, sinks and table tops) were collected for detection of *P. aeruginosa.* A performa which include age, gender, health status and relevant data were obtained from each patient, and the consent was taken from all patients before the collection of samples. Sterile cotton swabs (dipped in normal saline 0.9) were used to swab patients' burns and wounds and the surfaces of frequently handled items. Swabs were inoculated onto MacConky and blood agar plates (Oxoid and Himedia) and incubated at 37°C between 18 to 24 h. Colonies grown on culture plates were identified by the morphology of colonies, standard biochemical tests and gram staining (MacFaddin, 2000). Colonies that produce pyocyanin and pyoverdin pigments were confirmed by being transferred to nutrient agar (Oxoid, UK) and subcultured more than once to obtain pure cultures. *P. aeruginosa* isolates were identified using conventional biochemical tests such as oxidase test, catalase test, motility test, haemolysin production and other biochemical tests (Garcia and Isenberg, 2007; Elmer et al., 2006; Atlas and Synder, 2006).

Antibiotic susceptibility testing

The susceptibilities of the isolates to 14 antibiotics (Himedia, India): ticarcillin 75 µg, ceftazidime 30 µg, cefepime 30 µg**,** ofloxacin 5 µg, gentamycin 10 µg, tobramycin 10 µg, ticarcillin/clavulanate 75/10 µg, meropenem 10 µg, imepenem 10 µg, ciprofloxacin 5 µg, lomefloxacin 10 µg, norfloxacin 10 µg, levofloxacin 5 µg and amikacin 30 µg, were determined on Mueller-Hinton agar by the Kirby Bauer disk diffusion method. The zone of inhibition diameter was measured and the results were interpreted based on the guidelines by the Clinical and Laboratory Standards' institute CLSI 2011 and 2012.

Statistical analysis

The Microsoft Excel data analysis tool was used and the relevant data were collectively documented on a questionnaire and values expressed in means and percentage.

RESULTS

A total of 142 swab samples (92 from patients and 50 from hospital environment item) were collected for detection of *P. aeruginosa*. Among the total samples, 29 (20.42%) isolates of *the bacterium* were isolated; 18 (19.56%) from wounds and burns swabs of patients and 11 (22%) from hospital environment (Table 1). The distribution of *P. aeruginosa* isolates among different hospital environment items samples are shown in Table 2. The rate of *P. aeruginosa* was: 3(60%) in door handles, 4(57.15%) in ward sinks and 2 (10.53%) in both the patients' beds and table tops.

Table 3 demonstrated the distribution of *P. aeruginosa* according to age group and gender of patients. The results showed the high prevalence of *the bacterium* isolates among the male than female (55.6 and 44.4%, respectively), and the high rate of isolates were found among the age group 5 to 25 years (38.9%) and the low rate among age group >45 years (27.8%). Antibiotics

Table 2. Distribution of *P. aeruginosa* isolates among hospital environment specimens, 2012.

Specimen type	Specimens no.	*P. aeruginosa* isolates	
		No.	(%)
Patients beds	19	2	10.53
Door handles	5	3	60
Ward Sinks	7	4	57.15
Tables tops	19	2	10.53
Total	50	11	22

Table 3. Age and gender distribution of *P. aeruginosa* isolates for patients burns and wounds, 2012.

Variable	Total n=92	*P. aeruginosa* (n=18)	
		No.	%
Age/year			
5- 25	50	7	38.9
26-45	23	6	33.3
>45	19	5	27.8
Sex			
Female	38	8	44.4
Male	54	10	55.6

Table 4. Antibiotics resistance patterns of *P. aeruginosa* isolates.

Antibiotic	Clinical isolates (18)		Environmental isolates (11)	
	Resistant No.	Resistant %	Resistant No.	Resistant %
Ticarcillin 75 µg	18	100	11	100
Ceftazidime 30 µg	18	100	11	100
Cefepime 30 µg	16	88.8	10	90.9
Ofloxacin 5 µg	14	77.7	9	81.8
Norfloxacin 10 µg	7	38.8	5	45.4
Ciprofloxacin 5 µg	8	44.4	7	63.6
Levofloxacin 5 µg	7	38.8	5	45.4
Lomefloxacin 10 µg	8	44.4	6	54.5
Amikacin 30 µg	6	33.3	5	45.4
Tobramycin 10 µg	9	50,0	7	63.6
Gentamycin 10 µg	11	61.1	7	63.6
Ticarcillin/ clavulanate75/10 µg	3	16.6	3	27.2
Meropenem 10 µg	2	11.1	2	18.1
Imipenem 10 µg	1	5.5	1	9

susceptibility tests of *P. aeruginosa* against 14 different types of antibiotics is demonstrated in Table 4. All the 29 isolates screened from wounds and burns of patients and from hospital environment were resistant to ticarcillin and ceftazidime (100%). In *P. aeruginosa* from patients, resistance to cefepime, ofloxacin, gentamycin, tobramycin, ciprofloxacin, lomefloxacin, norfloxacin, levofloxacin and amikacin was 88.8, 77.7, 61.1, 50.0, 44.4, 44.4, 38.8, 38.8 and 33.3%, respectively. Whereas, the results showed that there was low resistance (16.6

and 11.1%) to each of ticarcillin/clavulanate and meropenem, and 5.5% of isolates were resistant to imepenem. Results showed that isolates of the bacterium from hospital environment were more resistant to antibiotics than those from the clinical environment. The level of resistance to cefepime, ofloxacin, gentamycin, tobramycin, ciprofloxacin, lomefloxacin, norfloxacin, levofloxacin, amikacin, ticarcillin/clavulanate, meropenem and imipenem were 90.9, 81.8, 63.6, 63.6, 63.6, 54.5, 45.4, 45.4, 45.4, 27.2, 18.1 and 9%, respectively.

DISCUSSION

P. aeruginosa is ranked second among gram-negative bacteria isolated from hospital environment, and leading cause of nosocomial infections responsible for high rate of morbidity and mortality (Meenakumari et al., 2011; Okon et al., 2009). The *bacterium* can cause serious infections in immunocompromised patients such as those with surgery wounds or severe burns (Sule et al., 2002; Dale et al., 2004). Result of this study showed that the prevalence of *P. aeruginosa* was 20.42% (29/142) among all the samples for patients and hospital environment, which is less than that reported in other studies; (39.1%) to that obtained by Okon et al. (2009) from wound swabs in Nigeria and 25.5% was reported in Cameron (Ndip et al., 2005). But these results were higher than 17.85% recorded in the teaching hospital of Al-Sulaimania city, Iraq (Ekrem et al., 2014) and 18.6% reported in Egypt (Gad et al., 2007). This disparity in prevalence rate among several studies can be attributed to differences in hygienic practices and geographical location.

The detection rate of *P. aeruginosa* in clinical samples 19.56% was not largely different from that of 22% from hospital environmental specimens and this result was similar to other studies in different area of the world (Ndip et al., 2005; Ekrem et al., 2014; Gad et al., 2007; Savaş et al., 2005). The results of this study agree with that of several other studies. (Meenakumari et al., 2011; Orsi et al., 1995; Sule et al., 2002) explain that *P. aeruginosa* is a common cause of infections in wound and burns contacted from the hospital environment or from patients own normal flora, and these infections leading to the longer hospital stays and increasing the treatment costs and mortality rate. The highest rate of *P. aeruginosa* which was identified from hospital environmental specimens in this study were from door handles (60%) followed with ward sinks (57.15%), This result could be explained by the fact that bacteria grow very well at sites with adequate amount of moisture and where people commonly come in contact with, while the result showed the least rate of *P. aeruginosa* (10.53%) in the patients' beds and tables tops, which are mostly kept dry. According to gender and age group, the result of this study shows the highest rate of *P. aeruginosa* in the male (55.6%), and 38.9% in the young patients (ages 5 to 25 years) compared to the elderly, while the lowest rate (27.8) was found among age group of forty five years and above, which indicates that males in this age group are more active and involve in different clinical hygiene practices, even in hospital environment. This result is comparable with the study of Okon et al in Nigeria, which recorded that male patients showed a record of 52.8% and the highest frequency of this bacterium (20.7%) was found in age group of 29 years and below (Okon et al., 2009). On the other hand, these results disagree with studies of Shewatatek et al. (2014) in Ethiopia and Ekrem and Rokan in Al-Sulaimania city, Iraq, where results of the studies showed higher occurrence of the bacterium in female and elderly patients (Shewatatek et al., 2014; Ekrem et al., 2014).

Results in Table 4 show the antibiotic susceptibility testing profile of *P. aeruginosa* that, 100% of isolates of clinical samples and hospital environment were resistance to ticarcillin and ceftazidime. The resistance of *P. aeruginosa* against the Beta-lactam antibiotics was higher than that of non Beta-lactam, this result can be attributed to the hyper production of Beta lactamase through the resistance genes and mutational processes (CDC, 2010; Lister et al., 2009; Okon et al., 2009). Also, *P. aeruginosa* isolates from patients demonstrated high resistance to cefepime, ofloxacin, gentamycin, tobramycin, ciprofloxacin, lomefloxacin, norfloxacin, levofloxacin and amikacin at 88.8, 77.7, 61.1, 50.0, 44.4, 44.4, 38.8, 38.8 and 33.3%, respectively, whereas the results showed low resistance (16.6 and 11.1%) to each of ticarcillin/clavulanate and meropenem, and only (5.5%) were resistant to imepenem. Generally, *P. aeruginosa* isolates from hospital environment were more resistant to particular antibiotics than that of clinical isolates. This result was similar with other studies done in Iraq (Ekrem et al., 2014), Egypt (Gad et al., 2007), Cameron (Ndip et al., 2005) and Nigeria (Okon et al., 2009), but differs with others such as study of Shewatatek et al. (2014) who recorded low resistance to these antibiotics. Difference in the resistance rate among several studies may be attributed to factors like hygienic culture of population, type of clinical specimen examined and exposure to antibiotics.

The resistance rate of Cefepime and ofloxacin in clinical and hospital environment isolates used in this study was recorded between 77 to 90.9%. This rate agree with the result of the study done in India (Prakash et al., 2012), but higher than that of Egypt (Gad et al., 2007) and Belgium (Van, 2003). Results in Table 4 also show that resistance rate to gentamycin was 61.1 and 63.6%, this finding is lower than those of studies reported in Egypt (67.5%) (Gad et al., 2007), Cameroon (66.7%) (Ndip et al., 2005) and Jordan (72%) (Masaadeh and Jaran, 2009). Whereas, the results are higher than the results of the study in Iraq (40%) (Ekrem and Rokan, 2014). The high resistance of *P. aeruginosa* isolates to gentamycin can be attributed to the wide use of this antibiotic in hospital

and the modification in bacteria enzymes. *P. aeruginosas* resistance to meropenem and imepenem was lower than other Beta-lactams used in the present study, in both patients and environment isolates. This result agrees with study of Ekrem and Rokan in 2014 that recorded 0% resistance rate to meropenem and imipenem in Iraq. The high sensitivity of these antibiotics can be attributed to low exposure because of the limited usage in Iraqi hospitals (Alzaidi and Alsulami, 2014). Meropenem and imipenem may be active therapy for *P. aeruginosa* infection.

Increasing resistance to different antibiotics especially among nosocomial pathogens has been reported world-wide and become important therapeutic challenge in the treatment of disease (Jones et al., 2002; Orrett, 2004). This fact agrees with the study of WHO which pointed that the widespread use of antibiotics both outside and inside of medicine is playing a significant role in the emergence of resistant bacteria by developing several resistance mechanisms such as production of Beta-lactamase enzymes that destroy these antibiotics (WHO, 2002). The major problem of the resistant bacteria emergence is due to overuse and misuse of antibiotics by patients as well as doctors (Goossens et al., 2005; Iduh et al., 2015), and may be related to random use of antibiotics without antibiotic sensitivity test and laboratory diagnosis.

Conclusion

The high rate and multidrug resistance of *P. aeruginosa* which were isolated from clinical and hospital environment probably occur as a result of wide use and abuse of antibiotics. Therefore the result of this study may be as a recommendation to the correct use of antibiotics in treatment of patients and also has to be considered as a part of infection control measures in hospital environment in order to reduce the risk of resistance development of *P. aeruginosa* infection.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Alzaidi JR, Alsulami AA (2014). Detection of methicillin resistant Staphylococcus aureus (MRSA) in the surgical wards by Chromogenic agar medium and PCR assay. Int. J. Biomed. Life Sci. 5(3):381-397.

Atlas RM, Snyder JW (2006). Handbook of media for clinical microbiology. 2nd ed. CRC Publisher. New York. Pp.278-339.

Brooks GF, Karen CC, Janet SB, Stephen AM (2011). Medical Microbiology. Jawetz, Melnick, & Adelberg. 25th edition, McGraw-Hill LANG. Chapter 16.

CDC-Centers for Disease Control and Prevention (2010). Antibiotic resistance questions and answers. Retrieved from http://www.cdcgov/getsmart/antibiotic-use/anitbiotic-resistance-faqs.html.

CLSI-Clinical and Laboratory Standards Institute (2011). Performance standards for antimicrobial susceptibility testing. Twenty-first Information supplement, CLSI document M100-S21. 31(1); M02-A10, M07-A8, Wayne PA.USA.

CLSI-Clinical and Laboratory Standards Institute (2012). Performance standards for antimicrobial susceptibility testing. Twenty-second Information supplement, CLSI document M100-S22. 32 (3); M02-A11, M07-A9, with M23-A3, M39-A3,M45-A2, Wayne PA.USA.

Dale RMK, Schnell G, Wong JP (2004). Therapeutic efficacy of antibiotics against burn wound infection by *Pseudomonas aeruginosa* Antimicrob. Agents Chemother. 48(8):2918-2923.

Ekrem K, Rokan DK (2014). Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* strains isolated from various clinical specimens. Sky J. Microbiol. Res. 2(2):13-17.

Elmer K, Washington W, Stephen A Jr., William J, Gary P, Paul S, Gail W (2006). Konemans color atlas and textbook of diagnostic microbiology. Sixth ed. Lippincott Williams and Wilkins, London.

Falagas ME, Bliziotis IA, Kasiakou SK, Samonis G Athanassopoulou P, Michalopoulos A (2005). Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria. BMC Infect. Dis. 5:24.

Gad GF, Domany RA, Zaki S, Ashour HM (2007). Characterization of *Pseudomonas aeruginosa* isolated from clinical and environmental samples in Minia, Egypt: prevalence, antibiogram and resistance mechanisms. J. Antimicrob. Chemother. 60:1010-1017.

Garcia LS, Isenberg HD (2007). Aerobic bacteriology. Clinical microbiology procedures handbook. 2nd ed. update, ASM Press. Washington C, D. 3:1.

Goossens H, Ferech M, Vander Stichele R, Elseviers M (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. Lancet 365(9459):79-87.

Haghi M, Maadi H, Delshad R, Nezhady M, Golizade S (2010). Antibiotic resistance pattern of *Escherichia coli Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from burnt patients Urmia, Iran. Int. J. Acad. Res. 2:377-380.

Iduh UM, Chollom CS, Nuhu A, Spencer TH, Nura MB, Ashcroft OF (2015). Nosocomial infections in post-operative wounds due to *Staphylococcus aureus* and Pseudomonas aeruginosa in Benue State Nigeria. Afr. J. Microbiol. Res. 9(36):1989-1996.

Jones RN, Kirby JT, Beach ML, Biedenbach DJ, Pfaller MA (2002). Geographic variations in activity of broad-spectrum lactams against *Pseudomonas aeruginosa*: Summary of the worldwide SENTRY antimicrobial surveillance program 1997–2000. Diagn. Microbiol. Infect. Dis. 43:239-243.

Lister PD, Wolter DJ, Hanson ND (2009). Antibacterial-Resistant *Pseudomonas aeruginosa:* Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin. Microbiol. 22(4):582-610.

MacFaddin JF (2000). Biochemical tests for identification of medical bacteria. Baltimore, MD: Lippincott Williams and Wilkins.

Masaadeh HA, Jaran AS (2009). Incident of *Pseudomonas aeruginosa* in post-operative wound infection. Am. J. Infect. Dis. 5:1-6.

Meenakumari S, Verma Sh, Absar A, Chaudhary A (2011). Antimicrobial susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* in an Indian cardiac hospital. Int. J. Eng. Sci. Technol. 3:7117-7124.

Ndip RN, Dilonga HM, Ndip LM, Akoachere JF, Akenji TN (2005). *Pseudomonas aeruginosa* isolates recovered from clinical and environmental samples in Buea, Cameroon: current status on biotyping and antibiogram. Trop. Med. Int. Health 10:74-81.

Okon K, Agukwe P, Oladosu W, Balogun S, Uba A (2009). Antibiotic resistance pattern of *Pseudomonas aeruginosa* isolated from clinical specimens in a tertiary hospital in northeastern Nigeria. J. Microbiol. 8(2):5-7.

Orrett FA (2004). Antimicrobial susceptibility survey of *Pseudomonas aeruginosa* strains isolated from clinical sources. J. Natl. Med. Assoc. 96(8):65-69.

Orsi GB, Tomao P, Visca P (1995). In vitro activity of commercially manufactured disinfectants against *Pseudomonas aeruginosa*. Eur. J. Epidemiol. 11:453-457.

Prakash HR, Rashmi B, Neena K, Suresh S, Anitha MR5, Vijayanath V

(2012). Antimicrobial susceptibility pattern of *Pseudomonas aeruginosa* strains isolated from clinical sources. J. Pharm. Biomed. Sci. 14:5.
Savaş L, Duran N, Savaş N, Onlen Y, Ocak S (2005). The prevalence and resistance patterns of *Pseudomonas aeruginosa* in intensive care units in a University hospital. Turk. J. Med. Sci. 35:317-322.
Shewatatek G, Gizachew T, Molalegne B, Terefe G (2014). Drug sensitivity of *Pseudomonas aeruginosa* from wound infections in Jimma university specialized hospital, Ethiopia. J. Med. Med. Sci. Res. 3(2):13-18.
Sule A, Olusanya O (2000). In-vitro antimicrobial activities of fluoroquinolones compared with common antimicrobial agents against clinical bacterial isolates from parts of South Western Nigeria. Niger. Q. J. Hosp. Med. 10(1):18-21.
Sule A, Thanni L, Sule-Odu O, Olusanya O (2002). Bacterial pathogens associated with infected wounds in Ogun state university teaching hospital, Sagamu, Nigeria. Afr. J. Clin. Exp. Microbiol. 3(1):13-16.
Van EJ (2003). Multicentre surveillance of Pseudomonas aeruginosa susceptibility pattern in nosocomial infection. J. Antimicrob. Chemother. 51:347-352.
WHO (2002). Humans. World Health Organization.

academicJournals

Vol. 10(23), pp. 850-855, 21 June, 2016
DOI: 10.5897/AJMR2016.7908
Article Number: 9062AF659078
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibacterial activity of silver-nanoparticles against *Staphylococcus aureus*

Mahsa Ydollahi[1], Hamed Ahari[1]* and Amir Ali Anvar[2]

[1]Department of Food Science and Technology, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Food Hygiene, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.

Received 6 January, 2016; Accepted 26 May, 2016

In this study, the antibacterial activity of silver nanoparticles (Ag-NPs) as Ag-nano disc was assessed with respect to Gram positive bacteria. Scanning electron microscope (ESM) was used for *Staphylococcus aureus* by measuring the diameter of inhibition zones in culture media and Ag-NPS. Eventually, gauzes containing strain were further impregnated between 2-layer nanofilms at 1, 3, 5 and 7 ppm sized 2 × 2 cm and placed inside the sterile Petri dish for each. The mean diameter (mm) of the inhibition zones surrounding the discs were significantly different (*p*= 0.000, F=5971.57) and varied from 2.41 to 6.19 mm and the bacteria *S. aureus* was sensitive to all Ag-NPs concentrations. The inhibited bacterial growth for 1 ppm of Ag nanodisk (2.51±0.01 mm) was less than 3 (3.21±0.02), 5 (4.27±0.01) as well as 7 ppm (6.00±0.02 mm). It was concluded that the best concentration of Ag nanodisc that can inhibit the growth of *S. aureus* is 7 ppm at the size of 80-120 mm for about 24 h after inoculation.

Keywords: *Staphylococcus aureus*, silver nanoparticles, antibacterial activity.

INTRODUCTION

With the occurrence and increase in bacterial resistance to most antibiotics and with emphasis on health cost, many investigators focus on low cost or free resistance of effective antimicrobial compounds (Jones et al., 2004). Such constraints have led health strategist to the renaissance in the use of Ag-based composites. The historian, Herodotus reported that Cyrus, the Great King of Persia from 559 to 530 B.C, had water drawn from a watercourse, had it boiled and carried in silver containers placed on numerous four-wheeled carriages drawn by mules that followed the king wherever he visited at any time (USEPA, 2012). Nanotechnology, an enabling technology involving the characterization and utilization of constitution or materials that are clusters of silver atoms ranging in diameter from 1 to 100 mm (Neethirajan and Jayas, 2011), is used as antibacterial in medical applications (Ozcalik and Tihminlioglu, 2013). Owing to inhibiting bacterial growth by foods with longer conservation, Ag-NPs (Ag-NPs) has been incorporated into a range of food contact equipment such as, keeping bags and fruit packages, but the medical industry has been slow to develop the property of Ag-NPs in infection

*Corresponding author. E-mail: Dr.h.ahari@gmail.com.

prophylaxis. However, it is currently used in an increasing number of consumer and medical applications (Chaloupka et al., 2010). Nanoparticles usually have been known to inhibit the bacterial growth and show better performance than the whole materials of the same elements in a major health problem. Silver ion has long been known to possess inhibitory and bactericidal activity on approximately 15 species containing bacteria (Feng et al., 2000). This potential could be due to the interaction between silver ion and the thiol group (sulphydryl-SH) of the bacterial cell membrane (Liau et al., 1997). The size of nanoparticles is very crucial in its bactericidal effect. The smaller the Ag-NPs size, the more the antibacterial activity increases. Small nanoparticles with a great contact area to volume ratio supply sufficient performance for the bactericidal property even at low concentration (Rai et al., 2009; Wijnhoven et al., 2009).

Silver sulfadiazine and silver nitrate have been widely used in superficial and deep dermal burns of wounds and for the removal of warts (Li et al., 2006; Wijnhoven et al., 2009). Silver is a more toxic metal to bacteria than many elements such as Ag >Hg >Cu>Cd>Cr >Pb> Co>Au>Zn>Fe (Zhao and Stevens, 1998).

Nanosilver shows a new generation of bactericidal and kills both Gram-negative and Gram-positive bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, (Sondi and Salopek-Sondi, 2004; Cho et al., 2005; Kim et al., 2011). It is also shown that chitosan nanoparticles and films have antimicrobial effects on *Listeria monocytogenes* and *S. aureus* (Rezaei and Kasra kermanshahi, 2013). *S. aureus* known as a facultative anaerobic gram-positive coccal bacterium is also an important cause of community and hospital-acquired infection. Methicillin-resistant infections caused by *S. aureus* are mainly nosocomial and are increasingly reported from many countries worldwide (Lowy, 1998). It is frequently part of the skin flora found in the nose and on the skin, and in this manner about 20% of the human population are long-term carriers of *S. aureus* (Ossowski et al., 2006). *S. aureus* is an important pathogen in the healthcare sector that has not been omitted from the hospital nor community environment. In humans, *S. aureus* causes superficial wounds in the skin and localized blisters, septicemia, septic arthritis, invasive endocarditis, and pneumonia (Ayala-Núñez et al., 2009).

The purpose of this study was to examine the antibacterial activity of different concentration of silver nanoparticles as antibiotic discs against *S. aureus* by measuring the diameter of inhibition zones in culture media.

MATERIALS AND METHODS

Silver nanofilms

Nanofilms as thin coating tangles infused with certain concentrations of 1, 3, 5 and 7 ppm silver compounds were produced after changing in the line of Nano Nasb Pars Company, Tehran, Iran. They were afterward transported to the laboratory and kept away from light for antimicrobial purposes.

Bacteria strain and measurement of colony-forming unit (CFU)

A Gram-positive bacteria, *S. aureus* with the concentration of 10° $CFU.mL^{-1}$ (ATCC 6538) was obtained from Iranian type Culture Collection to test the antibacterial activity of Ag nanocomposite film. The bacteria were kept at -80°C (stock solution), was cultured twice in Tryptic Soy Broth and incubated for 24-48 h at 37°C and consequently were sub-cultured on non-selective culture media, Tryptic Soy Agar and incubated for 24-48 h at 37°C to isolate the target colonies and suspended in saline solution (0.80% w/v). The bacterial suspension was attuned to the turbidity of McFarland standard solution 0.5, resulting in inoculums containing approximately 1×10^{8} CFU.mL.

A bacterial inoculum of *S. aureus* was sub-cultured in Baird Parker Agar (BP, Difco). Following this, discs (1 cm diameter) of each treatment for nanocomposite films were placed on the surface of the aforementioned inoculated agar culture media. Petri dishes with microorganism and discs of nanocomposite films were incubated at 37°C for 24 h. The antimicrobial activity of nanocomposite films was determined by measuring the inhibition zone around each disc of films (mm).

Disk diffusion assay

A disk diffusion method was used to assay the Ag-NPs for antibacterial activity against test strains. In the first stage, pure cultures of the bacteria were prepared in the liquid medium, buffered peptone water to ensure that the anti-bacterial property of nanofilms could be performed. The inoculates were prepared by diluting the overnight cultures with 0.9% NaCl to a 1 McFarland standard and were applied to the plates along with the standard. The sterile sample pieces of gauze with the definite size (2x2 cm) were prepared, allowed to soak into the liquid medium containing the bacteria on a rotary shaker (200 rpm) at 37°C and prepared disks containing differing amounts of Ag-NPs. Every piece of gauze soaked in pure culture and strain were further saturated between 2 layer nanofilms at 1, 3, 5 and 7 ppm sized 2x2 cm and each were placed inside the sterile Petri dish. Immediately, sterile swabs were carried out from the gauze samples and linearly culture was done on a nutrient agar medium. The same action (streaking) was carried out between 24 h intervals to 72 h. At that point they were incubated at 37°C for 24 h. Eventually, the results of Log_{10} CFU and the zones of inhibition (mm) were measured and recorded and the assays were performed in nine replicates (Lansdown, 2002).

Scanning electron microscope (SEM)

An SEM (Vega Tescan, USA) in which the reflected light determines the size of nano-particles that are invisible to the eye, was used in assaying the morphological measurement of nanoparticles. Master Batches or the constituent particles were dissolved in an acetonitrile solvent, to make a water base solution. These dissolved ingredients in the acetonitrile were put on two metal legs in a device called sputter coater. Nanoparticles were sputter coated with gold prior to the SEM examination (Thakur and SiṅGha, 2013).

Statistical analysis

All bacterial counts were expressed as log_{10} colony forming units per gram (log_{10} CFU). The mean log_{10} values were calculated on

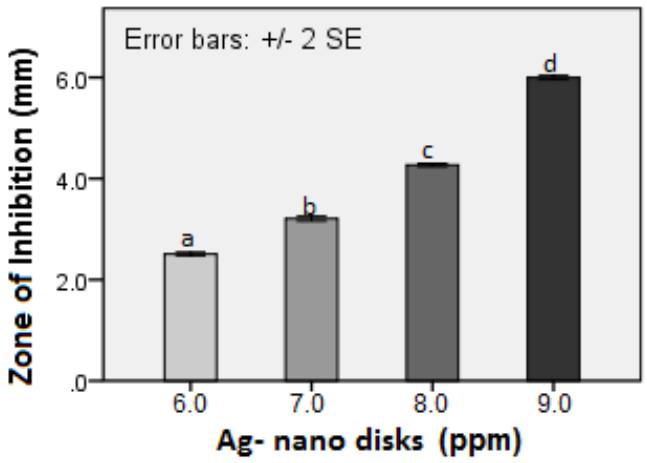

Figure 1. Zone of inhibition (mm) for antibacterial activity of different concentrations of Ag-nano discs against *S. aureus*.

the assumption of normal distribution. The effect of the nano-composite film at the different hours (Time × Nano) was analyzed with the GLM, Repeated Measurement ANOVA using SPSS 18. The greater and lower values of Pillai's Trace and Wilks' Lambda respectively showed more effectiveness in the independent factors (different concentrations of Ag nano film) on the bacteria inactivation while the data were analyzed at the level of α= 0.05. On the other hand, analysis of variance for the antibacterial action of the nanoparticles was carried out at different concentrations, by comparing the mean diameter of the inhibition zones. These measurements were also included measuring the frequency of the nano-particle size counted under the SEM.

Table 1. Estimated Marginal Means of $\log_{10}$ values of colony-forming units (n=9).

Ag-nano disc (ppm)	Time (h)	Mean±SE	95% confidence interval	
			Lower bound	Lower bound
Control	0	7.32±5.23	7.31	7.33
	24	7.51±4.91	7.52	7.52
	48	7.64±4.92	7.64	7.65
	72	7.74±5.26	7.75	7.75
1	0	7.32±5.23	7.31	7.33
	24	6.53±4.91	6.51	6.55
	48	7.66±4.92	7.66	7.66
	72	4.92±5.26	7.73	7.74
3	0	7.32±5.23	7.31	7.33
	24	5.49±4.91	5.14	5.68
	48	6.65±4.92	6.64	6.67
	72	7.74±5.26	7.74	7.74
5	0	7.32±5.23	7.31	7.33
	24	4.55±4.91	-5.14	5.32
	48	5.68±4.92	5.49	5.82
	72	7.76±5.26	7.76	7.77
7	0	7.32±5.23	7.31	7.33
	24	3.56±4.91	-5.23	5.25
	48	4.69±4.92	-5.09	5.35
	72	7.77±5.26	7.78	7.79

RESULTS

The results of preliminary test, multivariate statistical assess showed a strong and significant effects of independent variables, time (h) and concentration value of Ag nano disc (ppm) on dependent variable diameter of inhibition zone (mm) and counting the log $_{10}$ CFU of *S. aureus* (Pillai's Trace Value=2.90, Wilk's Lambda=0.00). The antibacterial effect of Ag-NPs as against *S. aureus* prepared on bacterial cultures and evaluation of their inhibitory diameters are shown in Figure 1.

According to the results of the antibacterial activity for the discs impregnated with 6, 7, 8 and 9 ppm of Ag-NPs, the mean diameter (mm) of the inhibition zones surrounding the discs was significantly different (*p*= .000, F=5971.57) from 2.41 to 6.19 mm with the bacteria *S. aureus* being sensitive to all Ag-NPs concentrations (Figure 1). The inhibited bacterial growth for 1 ppm of Ag-Nano disk (2.51±0.01 mm) was less than 3 ppm (3.21±0.02) and 5 ppm (4.27±0.01) as well as 7 (6.00±0.02 mm). Table 1 shows that the effect of interaction between the two variables (Time- nanosilver) on the measures of $\log_{10}$ CFU for *S. aureus.* Accordingly, the marginal means was significant for the 5 and 7 ppm at 24 and 48 h respectively, at which the estimated marginal mean values ($\log_{10}$) of CFU for the mentioned groups included zero at 95% confidence interval. However, the marginal mean value for the 7 ppm of Ag nanodisc (3.56±4.91) was obviously less than that of 5 ppm of Ag nanodisc (4.55±4.91) at 24 h. At 48 h, only its value for the 7 ppm of Ag nanodisc was significantly (*p*= .000) impressive and (*p*= .000) reached to 4.69±4.92 and increased to 7.77±5.26 at 72 h. As shown in Figure 3, the size of particles distributed with 15 kv and 5-10 kx magnifications was equal to 0.08 to 0.12 µm which infer on the size of 80-120 nm for the mean value of nano-particles in the discs.

DISCUSSION

An increasing trend of the inhibition zones diameter for the different Ag-nano concentrations from 1 to 7 ppm of Ag nano discs was seen. Based on the results given in Table 1, simultaneous interaction of the different concentrations of Ag nano discs (ppm) and time (h) were not significantly (*p*= .000) effective in reducing the marginal means of $\log_{10}$ values of CFU for groups,

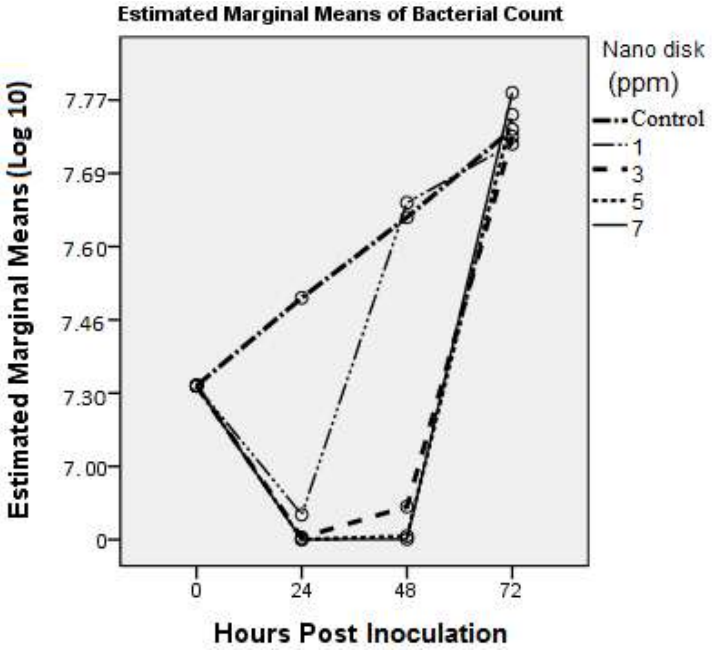

Figure 2. The effect of Ag-nano discs on estimated marginal means of log $_{10}$ CFU of *S. aureus.*

control, 1 and 3 ppm of Ag nano discs. An investigation (Rezaei and Kasra, 2013) representing a 3-log decrease from 6.0 at the beginning to 2.0 $\log_{10}$ CFU for about 16 h was constant till the end of the study (24 h), in exposing *S. aureus* with nanodisc of chitosan. This result is similar to our finding (marginal mean of $\log_{10}$ CFU) for 7 ppm of Ag nano disc for about 24 h but increased at 48 h in this study. Jung et al. (2008) showed reductions of more than 5 log10 CFU/ml of both *S. aureus* and *E. coli* bacteria that were confirmed after 90 min of treatment with the silver ion solution. Niakan et al. (2013) indicated that the zone of inhibition (mm) against *S. aureus,* with Ag-nanoparticles concentrations of 0.025 up to 0.0125 µg were 8.0 and 0.0 mm, respectively. Similar to our results, Shahverdi et al. (2007) confirmed the bactericidal property of Ag-NPs against *S. aureus* and on the other hand emphasized the presence of some antibiotics along with the Ag-NPs. Also, the results from a similar research (Kim et al., 2011) showed the minimum and more stable growth of *S. aureus* up to 8 h occurred with concentrations of 100 and 150 µg/ml of Ag-NPs. The results for Ag nano discs 5 and 7 ppm displayed significant (p= 0.000) decrease for the $\log_{10}$ CFU against *S. aureus* up to 24 and 48 h, respectively. Contrary to the present finding, Cho et al. (2005) indicated the antibacterial activity of Ag-NPs with 10 and 20 ppm is effective at 1 h after inoculation, being constant at the level of 3 logs CFU and reached 0.0 log CFU at the end of study (5 h). Accordingly, it showed that the stability property of Ag-NPs is remarkably dependent on the media culture PH while in this study and similar to Niakan et al. (2013), time was the important factor. In accordance with the result of Figure 2, the $\log_{10}$ CFU for 3, 5 and 7 ppm of Ag nano discs were effectively decreased at 24 h but statistically, the interaction between time and concentration of Ag-NPs was not significant for 1 ppm Ag-nano disc. Accordingly, the effectiveness of 5 and 7 ppm Ag-nano discs was remarkably decreased after 48 h, and only the interaction between time and concentration of Ag-NPs was significant for 7 ppm Ag-nano disc. The efficacy of the nano discs to decrease the log $_{10}$ marginal mean obviously decreased when it got to 72h. An investigation (Kim et al., 2007) was made on the effect of Ag-NPs on deactivating the yeast, *E. coli,* and *S. aureus*. Its results showed the inappropriate antibacterial activity for *S. aureus in contrary to* the yeast and *E. coli*, which is similar to our results that shows that the appropriate antibacterial activity for *S. aureus* was diminished after 24 h. This finding is also in agreement with the result of Guzman et al. (2012), which indicated Ag-NPs reasonable bactericidal activity against *E. coli*, *P. aeruginosa* and *S. aureus*. Also, Li et al. (2011) demonstrated that Ag-NPs passed the cell wall and interfered with the enzymatic formation. Subsequently, it entered the bacteria cell, condensed the DNA and prevented bacteria production.

Increasing the food life span using nano covers can be done by decreasing the size of materials lesser than 100 nanometer and that their properties follow the quantum

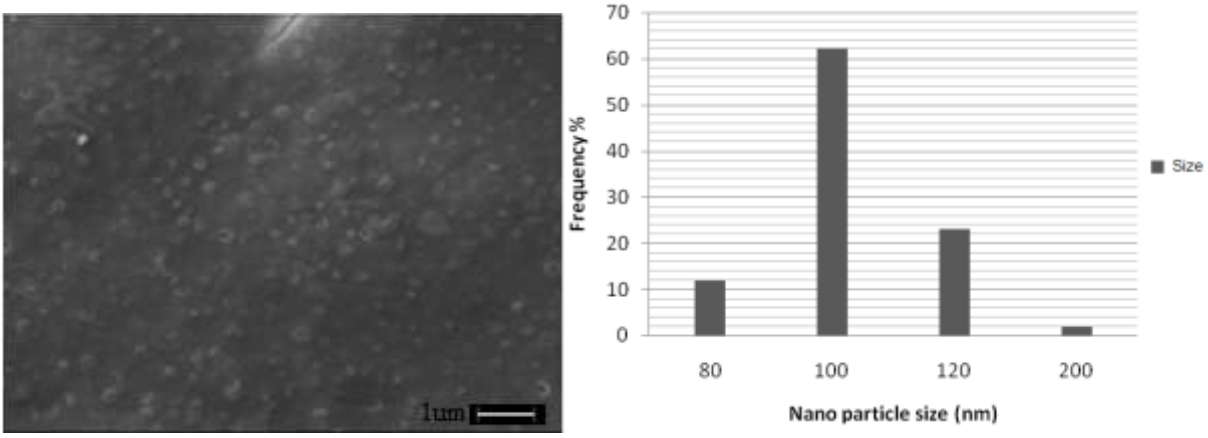

Figure 3. Left) Scanning electron microscope (SEM) of Ag-NPs, EHT=15.0, WD=10 mm, Mag. =10.0 kx; Particle size histogram of the silver particles is shown in the right hand picture.

physics rules, similar to the finding of Birla et al. (2009). These findings are in accordance with the results of Shahbazzadeh et al. (2011) who concluded that nanosilver has growth inhibiting on cancer cells two times more compared to natural and normal cells in the cases of Mesenchymal stem cells, natural fibroblast (HF2) and osteoblast (G292).

It is therefore concluded, the probability that the best concentration of Ag- nanodisc can inhibit the growth of *S. aureus* is 7 ppm at the size of 80-120 nm up to 24 h after inoculation and the present study results suggest that the efficacy of the Ag-NPs is decreased after the aforementioned time.

Conflict of interest

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENT

The authors thank the Head of Nano nasb co. for changing the production line of their factory to deliver the dedicated concentrations of Ag nano film for making the nano-silver disks.

REFERENCES

Ayala-Núñez NV, Villegas HHL, Turrent LdCI, Padilla CR (2009). Silver nanoparticles toxicity and bactericidal effect against methicillin-resistant *Staphylococcus aureus:* nanoscale does matter. Nanobiotechnology 5(1-4):2-9.

Birla S, Tiwari V, Gade A, Ingle A, Yadav A, Rai M (2009). Fabrication of silver nanoparticles by Phoma glomerata and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Lett. Appl. Microbiol. 48(2):173-179.

Chaloupka K, Malam Y, Seifalian AM (2010). Nanosilver as a new generation of nanoproduct in biomedical applications. Trends Biotechnol. 28(11):580-588.

Cho K-H, Park J-E, Osaka T, Park S-G (2005). The study of antimicrobial activity and preservative effects of nanosilver ingredient. Electrochim. Acta 51(5):956-960.

Feng Q, Wu J, Chen G, Cui F, Kim T, Kim J (2000). A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J. Biomed. Mater. Res. 52(4):662-668.

Guzman M, Dille J, Godet S (2012). Synthesis and antibacterial activity of silver nanoparticles against gram-positive and Gram-negative bacteria. Nanomedicine 8(1):37-45.

Jones SA, Bowler PG, Walker M, Parsons D (2004). Controlling wound bioburden with a novel silver-containing Hydrofiber® dressing. Wound Repair Regen. 12(3):288-294.

Jung WK, Koo HC, Kim KW, Shin S, Kim SH, Park YH (2008). Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*. Appl. Environ. Microbiol. 74(7):2171-2178.

Kim JS, Kuk E, Yu KN, Kim J-H, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang C-Y (2007). Antimicrobial effects of silver nanoparticles. Nanomedicine N.B.M. 3(1):95-101.

Kim SH, Lee HS, Ryu DS, Choi SJ, Lee DS (2011). Antibacterial activity of silver-nanoparticles against *Staphylococcus aureus* and *Escherichia coli*. Korean J. Microbiol. Biotechnol. 39(1):77-85.

Lansdown A (2002). Silver. I: Its antibacterial properties and mechanism of action. J. Wound Care 11(4):125-130.

Li W-R, Xie X-B, Shi Q-S, Duan S-S, Ouyang Y-S, Chen Y-B (2011). Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*. Biometals 24(1):135-141.

Li Y, Leung P, Yao L, Song Q, Newton E (2006). Antimicrobial effect of surgical masks coated with nanoparticles. J. Hosp. Infect. 62(1):58-63.

Liau S, Read D, Pugh W, Furr J, Russell A (1997). Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett. Appl. Microbiol. 25(4):279-283.

Lowy FD (1998). *Staphylococcus aureus* infections. New Engl. J. Med. 339(8):520-532.

Neethirajan S, Jayas DS (2011). Nanotechnology for the food and bioprocessing industries. Food Bioprocess Tech. 4(1):39-47.

Niakan M, Azimi HR, Jafarian Z, Mohammadtaghi G, Niakan S, Mostafavizade SM (2013). Evaluation of nanosilver solution stability against *Streptococcus mutans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Jundishapur J. Microbiol. 6(6).

Ossowski K, Chun RH, Suskind D, Baroody FM (2006). Increased isolation of methicillin-resistant Staphylococcus aureus in pediatric head and neck abscesses. Arch. Otolaryngol. Head Neck Surg. 132(11):1176-1181.

Ozcalik O, Tihminlioglu F (2013). Barrier properties of corn zein nanocomposite coated polypropylene films for food packaging

applications. J. Food Eng. 114(4):505-513.
Rai M, Yadav A, Gade A (2009). Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27(1):76-83.
Rezaei P, Kasra kermanshahi R (2013). Antimicrobial effects of chitosan nanoparticles and films on two species of foodborne pathogenic bacteria. J. Food Sci. Nutr. 13:5-12.
Shahbazzadeh D, Ahari H, Motalebi AA, Anvar AA, Moaddab S, Asadi T, Shokrgozar MA, Rahman-Nya J (2011). *In vitro* effect of Nanosilver toxicity on fibroblast and mesenchymal stem cell lines. Iran. J. Fish. Sci. 10(3):487-496.
Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian S (2007). Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. Nanomedicine: N.B.M. 3(2):168-171.
Sondi I, Salopek-Sondi B (2004). Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J. Colloid Interface Sci. 275(1):177-182.
Thakur VK, SiṅGha AS (2013). Biomass-based biocomposites. U. K., Smithers Rapra Technology.
USEPA (2012). Nanomaterial Case Study: N l Sil i Nanoscale Silver in Disinfectant Spray. USA, EPA. EPA/600/R-10/081F, 423 p.
Wijnhoven SW, Peijnenburg WJ, Herberts CA, Hagens WI, Oomen AG, Heugens EH, Roszek B, Bisschops J, Gosens I, Van De Meent D (2009). Nano-silver–a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3(2):109-138.
Zhao G, Stevens JSE (1998). Multiple parameters for the comprehensive evaluation of the susceptibility of *Escherichia coli* to the silver ion. Biometals 11(1):27-32.

academicJournals

Vol. 10(23), pp. 856-860, 21 June, 2016
DOI: 10.5897/AJMR2016.8002
Article Number: A9868A459080
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Seroepidemiology, molecular and entomological studies of bluetongue in sheep in Gujarat

Chauhan H. C.[1], Bhagat A. G. [2]*, Dadawala A. I. [2], Patel B. K. [1], Chandel B. S. [1], Kher H. N. [2], Patel M. A.[1] and Rajgor M.[2]

[1]Department of Animal Biotechnology, College of Veterinary Science and AH, S.D.A.U., Sardarkrushinagar- 385 506 (B.K.), Gujarat, India.
[2]Department of Microbiology, College of Veterinary Science and AH, S.D.A.U., Sardarkrushinagar- 385 506 (B.K.), Gujarat, India.

Received 10 March, 2016; Accepted 27 May, 2016

Seroepidemiology, molecular and entomological studies of bluetongue in sheep was carried out in Gujarat, India. Out of 980 sera sample screened, 361 (36.84 %) were found positive by c-enzyme-linked immunosorbent assay (c-ELISA). Out of the 103 blood samples tested for the presence of bluetongue virus (BTV) by reverse transcription-polymerase chain reaction (RT-PCR), 3 were found positive, producing 274 bp amplicons with NS1 gene specific primers. Collection and identification of *Culicoides* vectors from various farms suggest that the *Culicoides oxystoma* is the most prevalent species.

Key words: Bluetongue, sheep, c-Enzyme-linked Immunosorbent Assay (c-ELISA), NS1 *gene*, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), *Culicoides.*

INTRODUCTION

Bluetongue is an arthropod borne viral infection of domestic and wild ruminants. Although, all the ruminants are susceptible to bluetongue virus (BTV) infection, sheep is the most susceptible species to the clinical bluetongue disease. Bluetongue disease is a “List A” disease of the Office of International Epizootics (OIE). List A diseases are those diseases which can spread rapidly and the bluetongue is characterized by fever (42°C), hyperaemia, inflammation of the oral mucosa, tongue, coronaryband and occasionally the nasal mucosa, erosions and ulceration of the dermis and laminitis. Severe oedema of the tongue can result in restricted blood flow and cyanosis. The swollen tongue may protrude giving the appearance of a bluetongue. Sick animals may exhibit profuse salivation, depression, anorexia, weight loss due to muscle degeneration, stiffness of the limbs, lameness, and excessive nasal and ocular secretion. Death may occur in 8–10 days. The economic losses due to bluetongue is about 3 billion US$ per year in the world. The direct losses are death, abortions, weight loss and reduced milk and meat productions and indirect losses are export restrictions of live animals, semen and foetal calf serum (Bitew et al., 2013).

The causative agent, bluetongue virus (BTV) is a prototype virus of orbivirus of the family Reoviridae (Pringle, 1999). It is composed of 10 discrete segments of ds-RNA genome surrounded by two layers of protein capsid. Due to the presence of segmented RNA genome,

*Corresponding author. E-mail: abidalibhagat@gmail.com.

the virus is prone to frequent mutations. This has led to emergence of genomically diverse serotypes/strains of the virus. There are 26 BTV serotypes currently identified by the specificity of interactions between the outer capsid (particularly protein VP2) and the neutralizing antibodies generated during infection of the host (Maan et al., 2011). Out of 26 serotypes distributed globally, 21 serotypes have been reported from India (Wilson and Mellor, 2009). BTV is transmitted biologically by hematophagous midges in the genus *Culicoides* and infection occurs throughout the tropical and temperate regions of the world and coincident with the distribution of competent *Culicoides* vectors (Tabachnick, 1996).

Thus, the present study was carried out to determine the prevalence and distribution of BTV antibodies by c-enzyme-linked immunosorbent assay (c-ELISA), detection of BTV using reverse transcription-polymerase chain reaction (RT-PCR) and collection and identification of *Culicoides* vectors in various organized sheep farms of Gujarat.

MATERIALS AND METHODS

A total of 980 sera samples were collected for detection of BTV group specific antibodies by using BTV c-ELISA kit (Veterinary Diagnostic Technology Incorporation, USA) from sheep belonging to rural areas and organized farms of different districts of Gujarat state during the year 2008-2009. The separated serum was collected in screw capped plastic vial and heat inactivated at 56°C for 30 min. The sera were held at -20°C temperature till further use. The c-ELISA test was performed as described by Afshar et al. (1987). The data were analyzed by the Chi-square and odds ratio. The level of significance was set at $P< 0.05$.

A total of 103 blood samples were collected separately in vacutainers (Becton, Dickinson and Company) from sheep stationed at Gujarat Sheep and Wool Development Corporation (GSWDC), Jasdan and Aseda, LRS, Sardarkrushinagar and SBF, Patan. The samples were collected from sheep showing initial rise in body temperature (104°F) and showing clinical signs resembling BT. The samples were also collected from apparently healthy sheep. The blood samples on arrival at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, S.D. Agricultural University, Sardarkrushinagar were centrifuged at 800 *g* for 5 to 10 min in refrigerated centrifuge at 4°C. The blood cells were washed three times in equal volume of calcium magnesium free PBS. After the final wash, the equal volume of OPG medium was added in all the blood samples. The red blood cells were haemolysed as per the method described by Hosseini et al. (1998). After this, the blood samples were transferred in screw capped vials and stored at 4°C until further use. TRI RNA extraction method was used for total RNA isolation from blood samples as per the manufacturer's instruction (Molecular Research Centre, Inc., USA). The viral genomic RNA extracted by TRI reagent method was used as a template for cDNA synthesis by Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) enzyme (Bangalore Genei). The cDNA obtained was subjected to RT-PCR using partial length primer sequence for BTV group specific NS1 gene. The partial length PCR primers used were P1: 5'GTT CTC TAG TTG GCA ACC ACC3' and P2: 5' AAG CCA GAC TGT TTC CCG AT3' to generate an amplicon of 274 bp long. The NS1 gene based group specific PCR confirmed the samples as BTV. RT-PCR products where analyzed by agarose gel electrophoresis using 1.5% agarose gel prepared in 1X Tris-acetate-EDTA. The bands were visualized and photographed under Gel Documentation System (DNR Bio-imaging System) and photographed.

For the collections *Culicoides,* CDC miniature Insect Light Trap was employed close to animal sheds and operated from dusk to dawn (Figure 1). *Culicoides* were sorted out under a stereoscope and some *Culicoides* specimens were mounted and some were preserved in 70% alcohol for detailed identification. *Culicoides* collections were made from LRS, Sardarkrushinagar, GSWDC Sheep farm Jasdan and Aseda, SBF Patan. These way insects were collected, processed and finally sent to Centre for Animal Disease Research and Diagnosis, IVRI, Izatnagar for detailed identification.

RESULTS AND DISCUSSION

Presumptive diagnosis of BTV infection was made by serological test rather than by isolation and identification of the virus. To study the distribution and gravity of problem, seroepidemiology was considered as important tool and in the epidemiology of the bluetongue, and serological surveys are used to analyze the infection status of the ruminants in the area. The OIE manual of diagnostic tests and vaccines for terrestrial animals recommends AGID, c-ELISA and RT-PCR as prescribed tests for international trade. However, AGID is known for cross-reactions with other Orbiviruses such as epizootic haemorrhagicdisease virus (EDHV), thus its use has declined over time and replaced with the easier to use, rapid, highly sensitive and specific c-ELISA (Reddington et al., 1991).

Detection of virus specific antibodies in animals is an important criterion to study the epidemiology and provides an indirect evidence of virus circulation in a geographical area (Sreenivasulu and SubbaRao, 1999). Keeping the above facts in mind, a serological survey involving 980 sera sample from sheep was conducted using c-ELISA. Out of 980 sera sample screened, 361 (36.84%) were found to be positive (Table 1 and Figure 2). However, Sreenivasulu and SubbaRao (1999) reported higher rate of seroprevalence than this. Chandel et al. (2004) and Hinsu et al. (2000) recorded lower rate of 24.66 and 24.56% seroprevalence using Aar Gel Immuno-Diffusion (AGID) respectively, whereas Hinsu et al. (2000) reported a much higher rate of seroprevalence of 63.16% by c-ELISA from Gujarat State.

Out of 18 locations, the highest seroprevalence was recorded in GSWDC, Aseda while the lowest seroprevalence was recorded in Ahmedabad. The rate of seroprevalence in all these locations ranged between 27.59 and 44.29%. Univariate analysis showed that GSWDC, Aseda is more likely to be seropositive than Ahmedabad (OR=1.60), In contrast to the seroprevalence rate of 18.42% in Banaskantha, Chandel et al. (2004) reported 15.16% by BT-AGID and 32.00% in Kuchchh district; higher rate of seroprevalence, 59.64% by i-ELISA was reported by Bhalodiya and Jhala (2002). The reasons for this may be the variation in samples and place from where the sera were collected and tests

Figure 1. CDC miniature insect light trap employed at sheep farm.

employed for detection of BTV antibodies.

Sera were tested from five different regions *viz.,* North Gujarat, Saurashtra, Kuchchh, Central Gujarat and South Gujarat. Out of these five regions, the highest positivity was 40% in South Gujarat followed by North Gujarat Saurashtra, Kuchchh and Central Gujarat regions. Univariate analysis showed that South Gujarat is more likely to be seropositive than Central Gujarat, Kuchchh, Saurashtra and north Gujarat (OR=1.57, 1.40, 1.13 and 1.08, respectively). In contrast to the present findings, Bhalodiya and Jhala (2002) reported 69.23% from Saurashtra, and 59.69% from Kuchchh region of Gujarat State.

In the present study, four breeds of sheep were included. The rate of seroprevalence was highest in exotic (80%), followed by crossbred (53.68%), Patanwadi (35.09%) and Marwari (27.05%). The present findings revealed that exotic breeds are more susceptible than crossbreds and the indigenous breeds. Univariate analysis of breeds showed that exotic is 6 times more likely to be seropositive than Patanwadi (OR=6.98), also, analysis showed that exoticbreeds are 2 times more likely to be seropositive than Crossbred (OR=2.00) but Marwadi breed showed non-significant Chi-square value (0.1127, p<00000). Similar findings have been reported (Prasad et al., 1987; Das et al., 1997). However, on the contrary, Hinsu et al. (2000) found native sheep more susceptible than crossbred sheep for BTV infection. Higher rate of seroprevalence in crossbred than indigenous has also been reported by Sreenivasulu and SubbaRao (1999). Within indigenous breeds, Patanwadi showed higher rate of seroprevalence than Marwari, which is in accordance with the observations of Chandel et al. (2004).

Primer-directed amplification of viral nucleic acid has revolutionized BT diagnosis. It is one of the prescribed tests by OIE for international trade of animal products. This PCR assay may be used, not only to detect the presence of viral nucleic acid, but also for sero group orbiviruses and provide information on the serotype and possible geographical source of BTV isolates within a few days of receipt of clinical samples such as infected sheep blood. In the present study, Out of 103 blood samples tested for the presence of BTV by RT-PCR, 3 were positive

Table 1. Seroprevalence of BTV antibodies in sheep.

Attribute	Number of samples tested	No. positive	Percent positive
Region			
North Gujarat	570	217	38.07
Kuchchh	50	16	32.00
Saurashtra	266	98	36.84
Central Gujarat	74	22	29.73
South Gujarat	20	08	40.00
Location			
Banaskantha	200	83	41.50
Mehsana	95	35	36.84
Sabarkantha	20	07	35.00
Ahmedabad	29	08	27.59
Gandhinagar	15	05	33.33
Porbandar	17	05	29.41
Bharuch	20	08	40.00
Bhavnagar	23	08	34.78
Patan	70	24	34.29
SBF, Patan	50	19	38.00
LRS, Sardarkrushinagar	65	18	27.69
Jamnagar	56	17	30.36
Surendranagar	50	18	36.00
Amreli	25	09	36.00
Panchmahal	30	09	30.00
GSWDC, Jasdan	95	41	43.16
GSWDC, Aseda	70	31	44.29
Kuchchh	50	16	32.00
Breed			
Patanwadi	530	186	35.09
Marwadi	255	69	27.05
Crossbred	190	102	53.68
Exotic	05	04	80.00
Overall	980	361	36.84

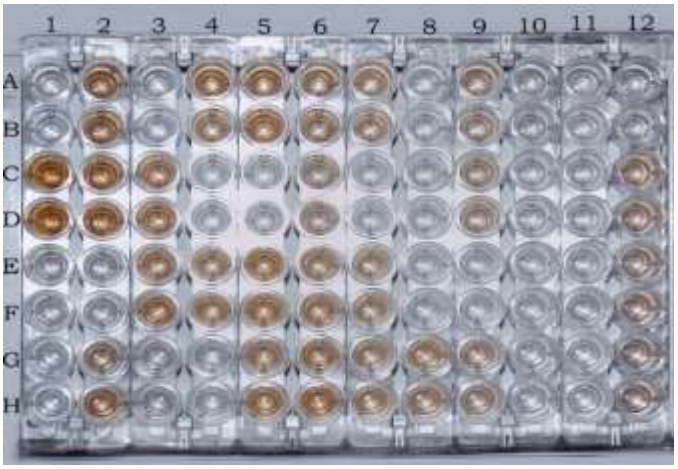

Figure 2. Microtitre ELISA plate showing results of c-ELISA. A1 and B1: Diluent control; C1 and D1: Negative control; E1 and F1: Weak positive control; G1 and H1: Positive control; A2 and B2; G12 and H12: Field sera.

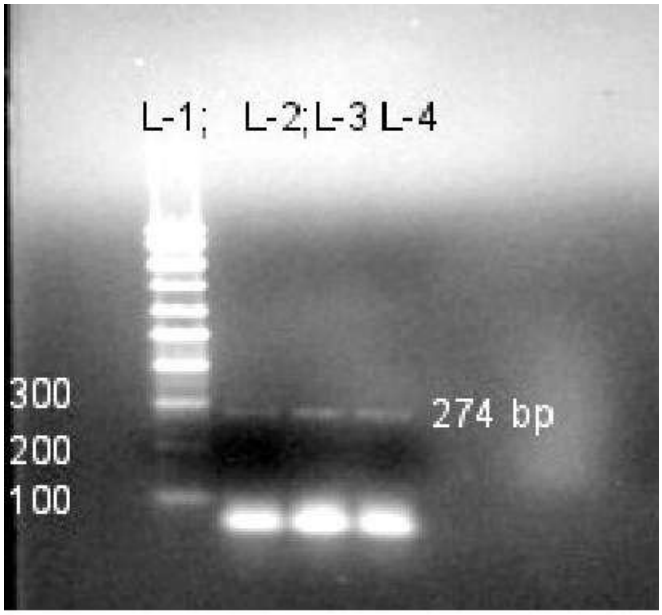

Figure 3. PCR product 274 bp by using BTV group specific nsl gene primer (L-1: 100 BP LADDER, Lane 2-4: Positive field samples).

for producing 274 bp amplicons with NS1 gene specific primers (Figure 3). Due to the conserved nature of NS1 gene, it was targeted for development of RT-PCR for detection of BTV. The NS1 gene primer sequence for partial length used in the study was the same as used by Katz et al. (1993). Similar, RT-PCR based amplification of 274 bp has also been carried out by Malik et al. (2001).

In the present study, collection and identification of *Culicoides* vectors from various farms suggest that the *C. oxystoma* is the most prevalent species and variable rate of seroprevalence has also been reported in these farms. As compared to the present study on collection and identification of Culicoides vectors, Patel et al. (2007) has also reported the prevalence of *Culicoides oxystoma* from LRS, Sardarkrushinagar; GSWDC, Jasdan; GSWDC, Aseda and LRS, Navsari which is in accordance with the results obtained in the present investigation. However, isolation of the virus from the trapped *Culicoides* and experimental transmission of the virus to the susceptible animals by vectors are required to confirm its role. Repeated trapping of a single species of *C. oxystoma* from sentinel herds/flocks, its close association with sheep and cattle and its continuous seroconversion in sentinel herds have been established in Punjab, Haryana, H.P. and Rajasthan (Kakkar et al., 2002). *Culicoides* are highly adapted to a wide range of temperature and moisture. In tropical areas, moisture is maintained by rain water, and in subtropical areas, it is through irrigation water. In South India, the monsoon season (June-December) with temperature ranging from 21.2 to 35.6°C appears to be favourable period for the multiplication of the vector resulting in more outbreaks. Bluetongue outbreaks in Karnataka, Tamil Nadu and Andhra Pradesh were associated with peak activity period of *Culicoides* spp.

Conclusion

In the present study, detection of BTV group specific antibodies and of BTV in blood samples by RT-PCR and identification of *C. oxystoma* from various farms indicate that the BTV is prevalent in Gujarat. Considering the immense economic importance of BTV and increasing trends of occurrence of BTV in India, potential vectors should be screened for identification, virus isolation and susceptibility of the infection and the virus transmission to vertebrate hosts. Forecasting of vector borne disease outbreaks through the development of appropriate models could be of great significance in controlling bluetongue disease.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by the All India Network Programme on Bluetongue Disease, Indian Council of Agricultural Research (ICAR), New Delhi, India. The authors acknowledge the Dean, College of Veterinary Science and A.H. S.D.A.U., Sardarkrushinagar for providing the facilities for the study.

REFERENCES

Afshar A, Thomas FC, Wright PF, Shapiro JL, Anderson J, Fulton RW

(1987). Blocking dot ELISA using a monoclonal antibody for detection of antibodies to bluetongue virus in bovine and ovine sera. J. Virol. Methods.18:271-280.
Bhalodiya MB, Jhala MK (2002). Seroepidemiological study of Bluetongue virus using AB-ELISA.Indian Vet. J. 79:1237-1240.
Bitew M, Nandi S, Ravishankar C, Somvanshi R (2013). Serological and molecular evidence of bluetongue in sheep and goats in Uttar Pradesh, India. Afr. J. Biotechnol. 12(19):2699-2705.
Chandel BS, KherHN, Chauhan HC, Vasava KA (2004). Serological survey of antibodies to bluetongue virus in domestic ruminants in Gujarat.Indian Vet. J. 81:737-740.
Das SC, Mishra S, Mishra N, Bhagwan PSK (1997). Comparison of competitive ELISA, indirect ELISA and agar gel precipitation test for the detection of Bluetongue virus antibodies in sheep. Indian J. Anim. Sci. 67:1033-1035.
Hinsu TV, Kher HN, Jhala MK, Kalyani IH (2000). Seroprevalence of bluetongue virus in Gujarat. IntasPolyvet. 1:216-218.
Hosseini M, Hawkes RA, Kirkland PD, Dixon RJ (1998). Rapid screening of embryonated chicken eggs for bluetongue virus infection with an antigen capture enzyme linked immunosorbent assay. J. Virol. Methods. 75:39-46.
Kakkar NK, Prasad G, Srivastava RN, Bhatnagar P (2002). Seroprevalence of Bluetongue virus infection in cattle in Haryana, Himachal Pradesh, Punjab and Rajasthan. Indian J. Comp. Immunol. Microbiol. Infect. Dis.23:147-151.
Katz JB, Gary AG, David AA, Karl AA, KathryanMM (1993). Colorimetric diagnosis of prolonged bluetongue viremia in sheep, using an enzyme linked oligonucleotide sorbent assay of amplified viral nucleic acids. Am. J. Vet. Res. 54:20-21.
Maan S, Maan NS, Nomikou K, Batten C, Antony F,Belaganahalli MN, Attia MS, Reda AA, Al-RashidSA, El Batel M, Oura CAL, Mertens PPC (2011). Novel bluetongue virus serotype from Kuwait. Emerg. Infect. Dis.17:886-889.
Malik Y, Prasad G, Minakshi, Maan S (2001). Evaluation of nucleic acid extraction methods for development of group specific nested PCR for identification of bluetongue virus. Indian J. Microbiol. 41:297-303.
Prasad G, Jain NC, Mahajan NK, Vasudevan B (1987). Prevalence of bluetongue precipitating antibodies in different domestic animals. Indian J. Anim. Sci. 57:522-524.
Pringle CR (1999). Virus taxonomy. Arch. Virol.144:421-429.
Reddington JJ, Reddington GM, MacLachlan NJ (1991). A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. J. Vet. Diag. Investig. 3:144-147.
Sreenivasulu D,SubbaRao MV (1999). Seroepidemiology of Bluetongue disease in Andhra Pradesh, India. Indian J. Anim. Sci. 69:292-294.
Tabachnick WJ (1996). *Culicoides variipennis* and bluetongue virus epidemiology in the United States. Ann. Rev. Entomol. 41:23-43.
Wilson AJ, Mellor PS (2009). Bluetongue in Europe: past, present and future. Philos. Trans. R. Soc. Biol. Sci. 364(1530):2669-2681.

academicJournals

Vol. 10(23), pp. 861-865, 21 June, 2016
DOI: 10.5897/AJMR2016.8024
Article Number: 5D9C89559084
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial activity of fixed oils found in Brazil nuts and sunflower seeds against microorganisms isolated from bovine subclinical mastitis

Cristina Hallal de Freitas[1]*, Carolina Lambrecht Gonçalves[2], Josiara Furtado Mendes[3], Deborah Murowaniecki Otero[4], Cláudio Martin Pereira de Pereira[1], Helenice de Lima Gonzales[5] and Patrícia da Silva Nascente[6]

[1]Programa de Pós-Graduação em Bioquímica e Bioprospecção, Universidade Federal de Pelotas, Pelotas, RS, Brasil.
[2]Programa de Pós-Graduação em Parasitologia, Universidade Federal de Pelotas, Pelotas, RS, Brasil.
[3]Programa de Pós-Graduação em Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brasil.
[4]Instituto Federal Farroupilha, Campus Jaguari, RS, Brasil.
[5]Faculdade de Veterinária Laboratório de Inspeção de Produtos de Origem Animal, Universidade Federal de Pelotas, Pelotas, RS, Brasil.
[6]Instituto de Biologia, Universidade Federal de Pelotas, Pelotas, RS, Brasil.

Received 23 March, 2016; Accepted 22 April, 2016

Concern with traces of antibiotics found in milk and resistant bacteria has led to the search for alternatives, mainly natural products, to treat mastitis. This study aimed at evaluating the antimicrobial activity of fixed oils found in Brazil nuts and sunflower seeds against microorganisms isolated from mastitic milk and at analyzing their fatty acids. The following microorganisms were isolated from milk produced by cows with subclinical mastitis and selected for the antimicrobial test: *Staphylococcus aureus*, *Enterococcus* spp., *Candida* spp. and *Cryptococcus laurentii.* The oil components and the antimicrobial activity were determined by gas chromatography and by the broth microdilution technique, respectively. The following fatty acids were identified: myristic, palmitic, palmitoleic, margaric, stearic, oleic, linoleic, linolenic, arachidic, gadoleic, behenic and lignoceric. Averages of minimum inhibitory concentrations (MIC) for *S. aureus*, *Enterococcus* spp., *Candida* spp. and *C. laurenti* were 36.30; 21.35; 15.62 and 10.41 µg.mL^{-1} (Brazil nut oil) and 23.5; 19.21; 7.81 and 1.2 µg.mL^{-1} (sunflower seed oil), respectively. These values show that these oils, in low concentration, have antimicrobial activity against the microorganisms under study. Therefore, this study shows that these fixed oils may be used as therapeutic resources for the control of mastitis.

Key words: Mastitis, antimicrobial activity, fixed oils.

INTRODUCTION

Mastitis, an inflammatory process in the mammary glands, causes major economic losses to dairy farms due to the decrease in milk production and quality, to the increase in medication use and to the risk of animal death. The etiology of this disease has shown that it may have toxic, traumatic, allergic, metabolic and mainly infectious origins (Melchior et al., 2006).

Even though several other microorganisms may affect

the intramammary region and cause an infection, *Staphylococcus aureus* is the main etiologic agent of chronic mastitis in dairy cows (Roberson et al., 1994). Despite the fact that bacteria are the agents which are more likely to be isolated, there are some cases of environmental microorganisms in the literature, mainly yeast from the genera *Candida* spp. and *Cryptococcus* spp. (Spanamberg et al., 2009).

Antibiotic therapies have been the most common procedures in the treatment of mastitis in dairy cows. However, concern with traces of antibiotics in milk and with resistant bacterial strains has led to the search for alternatives that may mitigate or eliminate such problems (Freitas et al., 2005). Researchers have become increasingly interested in natural products as sources of new drugs, such as medicinal plants (Pinto et al., 2006).

Therefore, this study aimed at evaluating the antimicrobial activity of fixed oils found in Brazil nuts and sunflower seeds against microorganisms isolated from milk from mammary quarts with subclinical mastitis.

MATERIALS AND METHODS

Chromatographic

Chromatographic analyses of Brazil nut and sunflower seeds oils were carried out by a GC-2010 chromatographer (Shimadzu, Japan) with an Elite-WAX capillary column (0.25 µm x 30 m x 0.25 mm). The injection of 1 µL of the sample was carried out in split mode (1:25). The carrier gas was H_2 (1.2 $mL.min^{-1}$). The initial temperature of the column was 140°C for 5 min; then, it was increased to 4°C per minute, up to 230°C for 10 min.Quantification was performed by the area normalization method proposed by GCSolution software whereas the identification of fatty acids was carried out by comparison with FAME MIX 37 standards (Sigma-Aldrich).

Antimicrobial tests

In this study, microorganisms that had been previously identified by the Vitek 2 system were selected: *S. aureus* (n=10), *Enterococcus* spp. (n=4), *Candida* spp. (n=4) and *Cryptococcus laurentii* (n=3) from bovine subclinical mastitis. Before the realization of the tests, it was recommended that bacteria which had resistance *in vitro* to at least five antibiotics be used in the routine of veterinary medicine.

The determination of the minimum inhibitory concentrations (MIC) of the oils was carried out by the broth microdilution technique, in agreement with CLSI 2008 (protocols M7-A6 and M27-A3, for bacteria and yeasts, respectively), adapted to a phytopharmacological agent.

The oils were prepared with dimethylsulphoxide (DMSO) as a dispersing solvent. In order to carry out the bacterial test, sterile microdilution plates that were previously filled with 100 µL Müeller-Hinton broth; ten successive oil dilutions, whose concentration varied from 170.8 to 0.33 $µg.mL^{-1}$, were made. Afterwards, plates were inoculated with 50 µL of the bacterium to be tested. It was been prepared to comply with 0.5 turbidity in the McFarland scale and adjusted in a cultivation media. After incubation at 36°C for 18 h, the plates had their MIC determined. In order to determine their minimum bactericidal concentrations (MBC), an aliquot of 5 µL from the first well of the microplate on which there was growth and from two preceding ones – were planted on a plate with Brain Heart Infusion Agar and incubated for 24 h so that bacterial growth could be observed.

Regarding yeasts, ten successive dilutions of oils were made on the microdilution plates; concentrations varied from 83.3 to 0.16 $µg.mL^{-1}$ in RPMI media. The microplates were inoculated with 100.0 µL of the microorganism to be tested and incubated at 36°C for 48h. Then, the MIC was determined. In order to determine their minimum fungicidal concentrations (MFC), an aliquot of 5 µL - from the first well of the microplate on which there was growth and from two preceding ones - was planted on a plate with Sabouraud dextrose agar and incubated for 24 h so that yeast growth could be observed.

RESULTS

Results of the chromatographic analysis of Brazil nut and sunflower seed oils are shown in Table 1. The following fatty acids were found in the oils under study: myristic acid (C 14:0), palmitic acid (C 16:0), palmitoleic acid (C 16:1), margaric acid (C 17:0), stearic acid (C 18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3), arachidic acid (C20:0), gadoleic acid (C20:1n9), behenic acid (C22:0) and lignoceric acid (C24:0). Results showed that oleic acid (C18:1n9c) and linoleic acid (C18:2n6c) are the fatty acids with the highest concentrations in Brazil nut oil. The former is composed of 30.61%, whereas the latter is composed of 47.26% of Brazil nut oil. Myristic acid (C 14:0) is the one with the lowest concentration (0.044%) in the composition of the fatty acids found in the sample.

The MIC of the oils, in relation to the microorganisms under study are shown in Table 2. In the case of Brazil nut oil, the averages of these concentrations were 36.30; 21.35; 15.62 and 10.41 $µg.mL^{-1}$ for *S. aureus*, *Enterococcus* spp., *Candida* spp. and *C. laurentii*, respectively. In the case of the 19 isolated microorganisms, MIC was similar to MBC/MFC. Regarding the sunflower seed oil, MIC's were 23.5; 19.21; 7.81 and 1.2 $µg.mL^{-1}$ for *S. aureus*, *Enterococcus* spp., *Candida* spp. and *C. laurentii*, respectively. MBC/MFC of this oil was equal to MIC's of 18 microorganisms.

DISCUSSION

Venkatachalam and Sathe (2006) determined the fatty acids found in Brazil nut oil in 2006: their values were similar to the ones found in this study, that is, 45.43% of

*Corresponding author. E-mail: cristinahallal@live.com.

Table 1. Identification and concentration of fatty acids found in Brazil nut and sunflower seed oils.

Compound	Brazil nut oil (%)	Sunflower seed oil (%)
Myristic acid (C 14:0)	0.044	0.071
Palmitic acid (C 16:0)	13.467	5.791
Palmitoleic acid (C 16:1)	0.232	0.073
Margaric acid (C 17:0)	0.129	0.039
Stearic acid (C 18:0)	7.741	4.106
Oleic acid (C 18:1n9c)	30.606	35.805
Linoleic acid (C 18:2n6c)	47.256	53.638
Linolenic acid (C 18:3n3)	0.095	0.043
Arachidic acid (C 20:0)	0.194	0.244
Gadoleic acid (C 20:1n9)	0.070	0.096
Behenic acid (C 22:0)	0.099	0.0866
Lignoceric acid (C 24:0)	0.065	0.228
Total	100.00	100.00

Table 2. Minimum inhibitory concentrations and minimum bactericidal/fungicidal concentrations of fixed oils found in Brazil nuts and sunflower seeds.

Microorganism	Brazil nut oil		Sunflower seed oil	
	MIC ($\mu g.mL^{-1}$)	MBC/MFC (µg/mL) (µg/mL)	MIC ($\mu g.mL^{-1}$)	MBC/MFC (µg/mL)
S. aureus	42.71	42.71	21.35	21.35
	42.71	42.71	21.35	21.35
	21.35	42.71	21.35	21.35
	42.71	42.71	21.35	21.35
	85.42	85.42	42.71	42.71
	21.35	42.71	21.35	42.71
	21.35	21.35	10.68	21.35
	42.71	42.71	21.35	21.35
	21.35	21.35	42.71	42.71
	21.35	21.35	10.68	21.35
Enterococcus spp.	21.35	21.35	21.35	21.35
	21.35	21.35	21.35	21.35
	21.35	21.35	21.35	21.35
	21.35	21.35	21.35	21.35
Candida spp.	10.41	10.41	5.21	5.21
	20.83	20.83	5.21	5.21
	20.83	20.83	10.41	10.41
	10.41	10.41	10.41	10.41
Cryptococcus laurentii	10.41	10.41	0.65	0.65
	10.41	10.41	2.60	2.60
	10.41	10.41	0.33	0.33

linoleic acid and 28.75% of oleic acid. Other authors, who also studied the content of fatty acids in this oil, found similar results (Santos and Marin, 2005; Spanamberg et al., 2009). Gas chromatography was employed to quantify the content of fatty acids in both previously mentioned studies.

The sunflower seed oil has the same main compounds of the Brazil nut one, that is, oleic acid represents 35.80% and linoleic acid is 52.64% of the total amount of fatty acids. Margaric acid (C 17:0) comprises only 0.039% of this oil. It is worth mentioning that linoleic acid is the most abundant unsaturated fatty acid in both samples under study.

Studies reported in literature have shown that linoleic acid is able to inhibit the growth of *S. aureus*, since it affects the protein synthesis on cell walls and on the nucleic acids (Mandelbaum et al., 2003). MIC values were higher against *S. aureus* (36.6 and 23.5µg.mL^{-1} for Brazil nut and sunflower seed oils, respectively) for both fixed oils under study. Increase in the prevalence of multi-resistant *S. aureus* which causes bovine mastitis is a serious problem worldwide since antimicrobial agents have less effective and morbidity rates and costs in fighting the disease have increased. There is considerable genetic heterogeneity in natural populations of *S. aureus*. Besides, it is an important pathogen which causes diseases that result from food ingestion, that is, the intake of toxins; thus, it may lead to a public health problem (Zafalon et al., 2008). These facts explain the stimulus for the search for alternative means to reduce or eliminate such problems.

Several studies demonstrate the resistance profile of agents causing bovine mastitis against antimicrobial agents available in the market for the treatment of this disease, for example, in a study conducted by Laport et al. (2012), 49 *Staphylcoccus* spp. strains were isolated from bovine mastitis cases from 21 different dairy herds kept at farms in Southeast Brazil. Strains were analyzed for antimicrobial susceptibility. Fifty-nine percent of the bacteria strains were resistant to at least one of the drugs tested and 12.2% were classified as multiresistant.

MBC was equal to MIC in 80% of the isolated microorganisms in the Brazil nut oil and in 70% of the sunflower seed oil ones. It shows that the oils exerted bactericidal/fungicidal and bacteriostatic/fungistatic activities against these strains. In a study carried out by Aquino et al. (2010), the fixed oil of *Ocimum basilicum* had bactericidal and bacteriostatic activity against *S. aureus* strains. Five out of eight strains under study were inhibited and killed when MIC's and MBC's were 3.12 and 6.25 µg.mL^{-1}, respectively. All isolates selected for this work, in a previous study, presented *in vitro* resistance to at least five antibiotics, which does not mean the MICs can be compared between them, since each active ingredient has specific concentrations to act.

Table 2 shows that the fixed oils under study exerted activity against yeasts in low concentrations; they varied from 10.41 to 20.83 µg.mL^{-1} in the case of Brazil nut oil and from 0.33 to 10.41 µg.mL^{-1} in the case of sunflower seed oil. The activity fixed oils exert against these microorganisms has been emphasized due to the scarcity of antimycotic pharmacological agents available in an adequate way to treat mycotic mastitis, mainly when antimicrobial medicines often used to fight the bacterial disease are compared.

High sensitivity was observed for *Cryptococcus laurentii* when sunflower seed oil was used; average MIC and MFC were 1.2 µg.mL^{-1}.

Regarding isolated microorganisms of *Candida* spp., the oils under analysis also had inhibitory activity in low concentrations; this fact is relevant since *Candida* represents the most commonly isolated pathogen from infections found in mammary glands of dairy cows, among all yeasts (Santos and Marin, 2005; Wunder, 2007). Besides, these results are also important due to the development of resistance against azolic agents which may cure candidiasis (caused by this yeast) in animals (Cleff et al., 2012).

Given these results, it was evidenced that the oils studied showed antimicrobial activity against microorganisms isolated from mastitis milk in low concentrations. Considering the current problem of difficulty in control and treatment of bovine mastitis due to resistance of these pathogens to antimicrobials routinely used in the treatment of mastitis and the concern of the presence of residues of these drugs in milk, the data demonstrated the potential use of these oils as a therapeutic resource in the control of this disease.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Aquino LC, Santos GG, Trindade RC, Alves JÁ, Santos PO, Alves PB, Blank AF, Carvalho LM (2010). Atividade antimicrobiana dos óleos essências de erva-cidreira e manjericão frente a bactérias de carnes bovinas. Alim. Nutr. 21(4):529-535.

Cleff MB, Meinerz ARM, Madrid I, Fonseca AO, Alves GH, Meireles MCA, Rodrigues MRA (2012). Perfil de suscetibilidade de leveduras do gênero *Candida* isoladas de animais ao óleo essencial de *Rosmarinus officinalis* L. Rev. Bras. Plant. Med. 14(1):43-49.

Clinical and Laboratory Standards Institute (2008). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. M7-A6, 2008.

Freitas MFL, Pinheiro Junior JW, Stamford TLM, Rabelo SSA, Silva DR., Silveira Filho VM, Santos FGB, Mota RA (2005). Perfil de sensibilidade antimicrobiana in vitro de *Staphylococcus* coagulase positivos isolados de leite com mastite no agreste do Estado de Pernanbuco. Arq. Inst. Biol. 72(2):171-177.

Laport MS, Marinho PR, Santos OC, Almeida P, Romanos MT, Muricy G, Brito MA, Giambiagi-deMarval M (2012). Antimicrobial activity of marine sponges against coagulase-negative staphylococci isolates from bovine mastites. Vet. Microbiol. 155(2-4):362-368.

Mandelbaum SH, Di Santis EP, Mandelbaum MHS (2003). Cicatrização: conceitos atuais e recursos auxiliares – Parte II. Ann. Bras. Dermatol. 78:393-410.

Melchior MB, Fink-Gremmels J, Gaastra W (2006). Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. J. Vet. Med. B. 53(5):326-332.

Pinto EPP, Amorozo MCM, Furlan A (2006). Conhecimento popular sobre plantas medicinais em comunidades rurais de mata atlântica - Itacaré, BA, Brasil. Acta Bot. Bras. 20(4):751-762.

Roberson JR, Fox LK, Hancock DD, Gay JM (1994). Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. J. Dairy Sci. 77(11):3354-3364.

Santos RC, Marin JM (2005). Isolation of Candida spp. from mastitic bovine milk in Brazil. Mycopathologia159(5):251-253.

Spanamberg A, Sanches EM, Santurio JM, Ferreiro L (2009). Mastite micótica em ruminantes causada por leveduras. Cienc. Rural 39(1):282-290.

Venkatachalam M, Sathe SK (2006). Chemical Composition of Selected Edible Nut Seeds. J. Agric. Food Chem. 54(13):4705-4714.

Wunder Júnior EA (2007). Mastite bovina: avaliação microbiológica do leite, com ênfase nas leveduras isoladas de casos de mastite clinica e subclínica, na região do planalto médio-RS, em 2005 e 2006. 2007. (Dissertação Mestrado). Universidade Federal do Rio Grande do Sul; Porto Alegre.

Zafalon LF, Arcaro JRP, Nader Filho A, Ferreira LM, Castelani L, Benvenutoo F(2008). Investigation of the antimicrobial resistance patterns in *Sthaphylococcus aureus* isolated in the milking of cows in the lactation. Rev. Inst. Adolfo Lutz 67(2):118-125.


African Journal of
Microbiology Research
Related Journals Published by Academic Journals
■ African Journal of Biotechnology
■ African Journal of Biochemistry Research
■ Journal of Bacteriology Research
■ Journal of Evolutionary Biology Research
■ Journal of Yeast and Fungal Research
■ Journal of Brewing and Distilling
academicJournals