Volume 10 Number 39 21 October, 2016
ISSN 1996-0808

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) 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/

Editors

Prof. Stefan Schmidt
Applied and Environmental Microbiology
School of Biochemistry, Genetics and Microbiology
University of KwaZulu-Natal
Pietermaritzburg,
South Africa.

Prof. Fukai Bao
Department of Microbiology and Immunology
Kunming Medical University
Kunming,
China.

Dr. Jianfeng Wu
Dept. of Environmental Health Sciences
School of Public Health
University of Michigan
USA.

Dr. Ahmet Yilmaz Coban
OMU Medical School
Department of Medical Microbiology
Samsun,
Turkey.

Dr. Seyed Davar Siadat
Pasteur Institute of Iran
Pasteur Square, Pasteur Avenue
Tehran,
Iran.

Dr. J. Stefan Rokem
The Hebrew University of Jerusalem
Department of Microbiology and Molecular Genetics
Jerusalem,
Israel.

Prof. Long-Liu Lin
National Chiayi University
Chiayi,
Taiwan.

Dr. Thaddeus Ezeji
Fermentation and Biotechnology Unit
Department of Animal Sciences
The Ohio State University
USA.

Dr. Mamadou Gueye
MIRCEN/Laboratoire commun de microbiologie
IRD-ISRA-UCAD
Dakar, Senegal.

Dr. Caroline Mary Knox
Department of Biochemistry, Microbiology and Biotechnology
Rhodes University
Grahamstown,
South Africa.

Dr. Hesham Elsayed Mostafa
Genetic Engineering and Biotechnology Research Institute (GEBRI)
Mubarak City For Scientific Research
Alexandria, Egypt.

Dr. Wael Abbas El-Naggar
Microbiology Department
Faculty of Pharmacy
Mansoura University
Mansoura, Egypt.

Dr. Barakat S.M. Mahmoud
Food Safety/Microbiology
Experimental Seafood Processing Laboratory
Costal Research and Extension Center
Mississippi State University
Pascagoula,
USA.

Prof. Mohamed Mahrous Amer
Faculty of Veterinary Medicine
Department of Poultry Diseases
Cairo university
Giza, Egypt.

Editors

Dr. R. Balaji Raja
Department of Biotechnology
School of Bioengineering
SRM University
Chennai,
India.

Dr. Aly E Abo-Amer
Division of Microbiology
Botany Department
Faculty of Science
Sohag University
Egypt.

Editorial Board Members

Dr. Haoyu Mao
Department of Molecular Genetics and Microbiology
College of Medicine
University of Florida
Florida, USA.

Dr. Yongxu Sun
Department of Medicinal Chemistry and Biomacromolecules
Qiqihar Medical University
Heilongjiang
P.R. China.

Dr. Ramesh Chand Kasana
Institute of Himalayan Bioresource Technology
Palampur,
India.

Dr. Pagano Marcela Claudia
Department of Biology,
Federal University of Ceará - UFC
Brazil.

Dr. Pongsak Rattanachaikunsopon
Department of Biological Science
Faculty of Science
Ubon Ratchathani University
Thailand.

Dr. Gokul Shankar Sabesan
Microbiology Unit, Faculty of Medicine
AIMST University
Kedah,
Malaysia.

Editorial Board Members

Dr. Kamel Belhamel
Faculty of Technology
University of Bejaia
Algeria.

Dr. Sladjana Jevremovic
Institute for Biological Research
Belgrade,
Serbia.

Dr. Tamer Edirne
Dept. of Family Medicine
Univ. of Pamukkale
Turkey.

Dr. Mohd Fuat ABD Razak
Institute for Medical Research
Malaysia.

Dr. Minglei Wang
University of Illinois at Urbana-Champaign
USA.

Dr. Davide Pacifico
Istituto di Virologia Vegetale – CNR
Italy.

Prof. N. S. Alzoreky
Food Science & Nutrition Department
College of Agricultural Sciences & Food
King Faisal University
Saudi Arabia.

Dr. Chen Ding
College of Material Science and Engineering
Hunan University
China.

Dr. Sivakumar Swaminathan
Department of Agronomy
College of Agriculture and Life Sciences
Iowa State University
USA.

Dr. Alfredo J. Anceno
School of Environment, Resources and Development (SERD)
Asian Institute of Technology
Thailand.

Dr. Iqbal Ahmad
Aligarh Muslim University
Aligrah,
India.

Editorial Board Members

Dr. Juliane Elisa Welke
UFRGS – Universidade Federal do Rio Grande do Sul
Brazil.

Dr. Iheanyi Omezuruike Okonko
Department of Virology
Faculty of Basic Medical Sciences
University of Ibadan
Ibadan,
Nigeria.

Dr. Giuliana Noratto
Texas A&M University
USA.

Dr. Babak Mostafazadeh
Shaheed Beheshty University of Medical Sciences
Iran.

Dr. Mehdi Azami
Parasitology & Mycology Department
Baghaeei Lab.
Isfahan,
Iran.

Dr. Rafel Socias
CITA de Aragón
Spain.

Dr. Anderson de Souza Sant'Ana
University of São Paulo
Brazil.

Dr. Juliane Elisa Welke
UFRGS – Universidade Federal do Rio Grande do Sul
Brazil.

Dr. Paul Shapshak
USF Health
Depts. Medicine and Psychiatry & Beh Med.
Div. Infect. Disease & Internat Med
USA.

Dr. Jorge Reinheimer
Universidad Nacional del Litoral (Santa Fe)
Argentina.

Dr. Qin Liu
East China University of Science and Technology
China.

Dr. Samuel K Ameyaw
Civista Medical Center
USA.

Dr. Xiao-Qing Hu
State Key Lab of Food Science and Technology
Jiangnan University
China.

Prof. Branislava Kocic
University of Nis
School of Medicine
Institute for Public Health
Nis,
Serbia.

Prof. Kamal I. Mohamed
State University of New York
Oswego,
USA.

Dr. Adriano Cruz
Faculty of Food Engineering-FEA
University of Campinas (UNICAMP)
Brazil.

Dr. Mike Agenbag
Municipal Health Services,
Joe Gqabi,
South Africa.

Dr. D. V. L. Sarada
Department of Biotechnology
SRM University
Chennai
India.

Prof. Huaizhi Wang
Institute of Hepatopancreatobiliary
Surgery of PLA Southwest Hospital
Third Military Medical University
Chongqing
China.

Prof. A. O. Bakhiet
College of Veterinary Medicine
Sudan University of Science and Technology
Sudan.

Dr. Saba F. Hussain
Community, Orthodontics and Peadiatric Dentistry Department
Faculty of Dentistry
Universiti Teknologi MARA
Selangor,
Malaysia.

Editorial Board Members

Prof. Zohair I. F. Rahemo
Department of Microbiology and Parasitology
Clinical Center of Serbia
Belgrade,
Serbia.

Dr. Afework Kassu
University of Gondar
Ethiopia.

Dr. How-Yee Lai
Taylor's University College
Malaysia.

Dr. Nidheesh Dadheech
MS. University of Baroda,
Vadodara,
India.

Dr. Franco Mutinelli
Istituto Zooprofilattico Sperimentale delle Venezie
Italy.

Dr. Chanpen Chanchao
Department of Biology,
Faculty of Science,
Chulalongkorn University
Thailand.

Dr. Tsuyoshi Kasama
Division of Rheumatology,
Showa University
Japan.

Dr. Kuender D. Yang
Chang Gung Memorial Hospital
Taiwan.

Dr. Liane Raluca Stan
University Politehnica of Bucharest
Department of Organic Chemistry
Romania.

Dr. Mohammad Feizabadi
Tehran University of Medical Sciences
Iran.

Prof. Ahmed H Mitwalli
Medical School
King Saud University
Riyadh,
Saudi Arabia.

Dr. Mazyar Yazdani
Department of Biology
University of Oslo
Blindern,
Norway.

Dr. Babak Khalili Hadad
Department of Biological Sciences
Islamic Azad University
Roudehen,
Iran.

Dr. Ehsan Sari
Department of Plant Pathology
Iranian Research Institute of Plant Protection
Tehran,
Iran.

Dr. Snjezana Zidovec Lepej
University Hospital for Infectious Diseases
Zagreb,
Croatia.

Dr. Dilshad Ahmad
King Saud University
Saudi Arabia.

Dr. Adriano Gomes da Cruz
University of Campinas (UNICAMP)
Brazil

Dr. Hsin-Mei Ku
Agronomy Dept.
NCHU
Taichung,Taiwan.

Dr. Fereshteh Naderi
Islamic Azad University
Iran.

Dr. Adibe Maxwell Ogochukwu
Department of Clinical Pharmacy and Pharmacy Management,
University of Nigeria
Nsukka,
Nigeria.

Dr. William M. Shafer
Emory University School of Medicine
USA.

Dr. Michelle Bull
CSIRO Food and Nutritional Sciences
Australia.

Editorial Board Members

Prof. Márcio Garcia Ribeiro
School of Veterinary Medicine and Animal Science-UNESP,
Dept. Veterinary Hygiene and Public Health,
State of Sao Paulo
Brazil.

Prof. Sheila Nathan
National University of Malaysia (UKM)
Malaysia.

Prof. Ebiamadon Andi Brisibe
University of Calabar,
Calabar,
Nigeria.

Dr. Julie Wang
Burnet Institute
Australia.

Dr. Jean-Marc Chobert
INRA- BIA, FIPL
France.

Dr. Zhilong Yang
Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases,
National Institutes of Health
USA.

Dr. Dele Raheem
University of Helsinki
Finland.

Dr. Biljana Miljkovic-Selimovic
School of Medicine,
University in Nis,
Serbia.

Dr. Xinan Jiao
Yangzhou University
China.

Dr. Endang Sri Lestari, MD.
Department of Clinical Microbiology,
Medical Faculty,
Diponegoro University/Dr. Kariadi Teaching Hospital,
Semarang
Indonesia.

Dr. Hojin Shin
Pusan National University Hospital
South Korea.

Dr. Yi Wang
Center for Vector Biology
Rutgers University
New Brunswick
USA.

Prof. Natasha Potgieter
University of Venda
South Africa.

Dr. Sonia Arriaga
Instituto Potosino de Investigación Científicay Tecnológica/
División de Ciencias Ambientales
Mexico.

Dr. Armando Gonzalez-Sanchez
Universidad Autonoma Metropolitana Cuajimalpa
Mexico.

Dr. Pradeep Parihar
Lovely Professional University
Punjab,
India.

Dr. William H Roldán
Department of Medical Microbiology
Faculty of Medicine
Peru.

Dr. Kanzaki, L. I. B.
Laboratory of Bioprospection
University of Brasilia
Brazil.

Prof. Philippe Dorchies
National Veterinary School of Toulouse,
France.

Dr. C. Ganesh Kumar
Indian Institute of Chemical Technology,
Hyderabad
India.

Dr. Zainab Z. Ismail
Dept. of Environmental Engineering
University of Baghdad
Iraq.

Dr. Ary Fernandes Junior
Universidade Estadual Paulista (UNESP)
Brasil.

Editorial Board Members

Dr. Fangyou Yu
The first Affiliated Hospital of Wenzhou Medical College
China.

Dr. Galba Maria de Campos Takaki
Catholic University of Pernambuco
Brazil.

Dr Kwabena Ofori-Kwakye
Department of Pharmaceutics
Kwame Nkrumah University of Science & Technology
Kumasi,
Ghana.

Prof. Liesel Brenda Gende
Arthropods Laboratory,
School of Natural and Exact Sciences,
National University of Mar del Plata
Buenos Aires,
Argentina.

Dr. Hare Krishna
Central Institute for Arid Horticulture
Rajasthan,
India.

Dr. Sabiha Yusuf Essack
Department of Pharmaceutical Sciences
University of KwaZulu-Natal
South Africa.

Dr. Anna Mensuali
Life Science
Scuola Superiore Sant'Anna
Italy.

Dr. Ghada Sameh Hafez Hassan
Pharmaceutical Chemistry Department
Faculty of Pharmacy
Mansoura University
Egypt.

Dr. Kátia Flávia Fernandes
Department of Biochemistry and Molecular Biology
Universidade Federal de Goiás
Brasil.

Dr. Abdel-Hady El-Gilany
Department of Public Health & Community Medicine
Faculty of Medicine
Mansoura University
Egypt.

Dr. Radhika Gopal
Cell and Molecular Biology
The Scripps Research Institute
San Diego, CA
USA.

Dr. Mutukumira Tony
Institute of Food Nutrition and Human Health
Massey University
New Zealand.

Dr. Habip Gedik
Department of Infectious Diseases and Clinical Microbiology
Ministry of Health Bakırköy Sadi Konuk Training and Research Hospital
Istanbul,
Turkey.

Dr. Annalisa Serio
Faculty of Bioscience and Technology for Food Agriculture and Environment
University of Teramo
Teramo,
Italy.

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 39 21 October, 2016

ARTICLES

Effectiveness of augmented consortia of *Bacillus coagulans*, Citrobacter koseri and *Serratia ficaria* in the degradation of diesel polluted soil supplemented with pig dung 1637
Fagbemi O. Kehinde and Sanusi A. Isaac

Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* isolates from gyptian hospitals 1645
Marwa M. Raafat, Marwa Ali-Tammam and Amal E. Ali

Bacterial association and oral biofilm formation: A bibliometric analysis 1654
Luana Kelle Batista Moura, Fabrício Ibiapina Tapety, Mitra Mobim,
Eliana Campelo Lago, Eduardo Souza de Lobão Veras,
arla Maria de Carvalho Leite Leal Nunes,
Tanit Clementino Santos and Thiago Lima Monte

Campylobacter in sheep, calves and broiler chickens in the central region of Algeria: Phenotypic and antimicrobial resistance profiles 1662
Meryem Guessoum, Zehor Guechi, Fares Aigoun, Sadjia Mahrane and Amina Hachemi

Effects of immunoglobulin Y (IgY) serum against plaque bacteria 1668
Juni Handajani, Dhinintya Hyta Narissi, Mufidana Azis and Aurita Siwi Rahmawati

academicJournals

Vol. 10(39), pp. 1637-1644, 21 October, 2016
DOI: 10.5897/AJMR2016.8249
Article Number: BFAEEFF61243
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effectiveness of augmented consortia of *Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria* in the degradation of diesel polluted soil supplemented with pig dung

Fagbemi O. Kehinde[1] and Sanusi A. Isaac[2]*

[1]The Department of Microbiology, University of Lagos, Akoka, Lagos State, Nigeria.
[2]Discipline of Microbiology, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa.

Received 3 August 2016, Accepted 11 October, 2016

Laboratory studies were developed to compare the effectiveness of inoculated bacteria consortia and indigenous microorganisms on diesel-polluted soil for 18 days. Bacteria isolated from the unpolluted soil sample were: *Pseudomonas* spp. (LBl), *Pseudomonas cepacia* (LB5), *Micrococcus luetus* (LB2), *Bacillus subtilis* (LB3) and *Bacillus cereus* (LB4). Their ability to degrade different substrates were first studied by the presence of growth in minimal salt broth. All the isotates were unable to grow in hexane. LB1 and LB2 had a strong growth in n-dodecane and n-hexadecane. Only LB1, LB4 and LB5 were able to grow in paraffin. LB1 and LB5 had poor growth on xylene. LB1 and LB3 had moderate growth in phenol. All the isolates had little growth in kerosene and only LB3 and LB4 grow in diesel. The three most promising of the isolates, with moderate to strong growth (LB2, LB4 and LB5) on crude oil were further used for diesel bioremediation. The bacterial population in the augmented diesel-contaminated soil showed a reduction in the population density from days 15 to 18, an indication of nutrients (diesel oil) exhaustion. While the un-augmented diesel-polluted soil samples showed potential of more days of increased bacterial population after the 18th day of observation, a pointer of more diesel in the soil samples that can be metabolized/utilized by the microorganisms present in soil samples. The consortia of *Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria* was effective in the removal of 73.8% diesel oil from the diesel-polluted soil sample while natural attenuation resulted in 41.0% diesel oil removal and 35.8% in the control.

Key words: Bioaugumentation, consortia, diesel, effectiveness, pig dung, soil.

INTRODUCTION

Oil producing conutries of the world are, faced with challenges related to rehabilitation of polluted

*Corresponding author. E-mail: Sanusi_isaac@yahoo.com. Tel: +2761618162.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

environments, leading to the development of a wide range of clean up techniques including physical, chemical and biological methods (Wang et al., 1994, Rui et al., 2012). The first two categories hardly achieve holistic elimination of oil contamination from environments with second method involving application of expensive chemical dispersants introducing even more pollutants (Jain et al., 1992; Esin and Ayten, 2011). This makes biological method indispensible as the most natural method to eliminate the bulk of oil contaminants from the environment. The biological method exploits the diverse degradation abilities of microorganisms to convert the complex chemical components of crude oil to harmelss products by mineralization (Chandran, 2011; Chemlal et al., 2013). The acute need for energy in the world today has resulted in a large output of oil and oil products; thus, much of the hydrocarbon material that is extracted from the earth in various parts of the world is transported to other parts of the world for refining. A measure of the oil, both in crude form and in various refined forms is lost to the environment, particularly accidental spills. Terrestial oil spills arise from production, transportation and storage accidents. From these, pipeline failures are the most likely ones to inundate agricultural or wilderness areas (Roy et al., 2014). Diesel oil, sometimes called fuel oils, is classified as middle distillates of crude oil and consists of hydrocarbons with number of carbons atoms mainly in the range of C_9 – C_{20}. Although, the proportion of diesel fuel that maybe subjected to volatilization (C_2 – C_{10}) is small (1500 – 6300 µg g^{-1}), some of these volatilized hydrocarbon are toxic and can cause health risk. It is known that oil pollution can occur naturally, like the tar sands in Alberta, where oil has worked its way to the surface (Mielke, 1990; Erin et al., 2010; Roy et al., 2014). However, this sources seems minute as compared to the oil spilled by man into the soil through routine leaks from tanks, from oil rigs and pipelines and the most sensational of all supertanker accidents. Oil spills have many adverse effects on the environment. When oil spills occur, the domestic, agricultural and industrial uses are impaired (Ibrahim et al., 2008; Roy et al., 2014, Abu et al., 2016). The cummulative impact of repeated small oil spills can devastate the environment. Elimination of these small oil spills is essential to ensuring the health and productivity of our agricultural lands, not only for us but for future generations. In Nigeria, it takes a long time for arable land polluted with crude oil to regain its fertility. Thus, the amount of compensation paid to farmers by polluters (mainly oil industries and government agencies) is sometimes grossly under estimated, consindering the damage done to the soil fertility and the expected produce (Nwachukwu, 2000). Biological reactions involved in the degradation of petroleum are also important in natural systems. In general, it can be assumed that when crude oil is discharged into a system, those fractions with boiling points less than 370°C will evaporate from the system in a matter of days. This leaves biological and autocatalytic decomposition to operate on the remaining fraction. An important concept to remember at this point is that biological decay usually involves only specific compounds. Crude oil is a complex mixture of hydrocarbons, many of which are toxic to be tolerated and degraded (Bragg, 1994; Abu et al., 2016). Thus, when microorganisms attack crude oil, certain fractions are utilized preferentially and certain fractions remain. Van Hammed et al. (2003) listed the general hierarchy of hydrocarbons with respect to preference for microbial degradation. They suggested that alkanes are more readily degraded than aromatic hydrocarbons and that within the alkane's straight-chain compounds are more susceptible to microbial action than branching chains. Methane, ethane and propane are attacked by only a few highly specialized organisms and the more refractory materials, such as waxes and compounds containing more than 30 carbon atoms are insoluble and therefore highly resistant to degradation (Bossert and Compeau, 1995; Yousseria et al., 2016). Biodegradation is a biologically catalyzed oxidation or reduction reaction involving complex chemical compounds. This process can be based on either growth (organic pollutants are used as the sole source of carbon and energy) or co-metabolism. Co-metabolism is the breaking down of organic compounds in the presence of a growth substrate which is used as the primary carbon and energy source (Das and Chandran, 2011). These microbial activities occur with effective cooperation from the soil (Laleh et al., 2016). Biological degradation of hydrocarbons in the environment is also linked to a number of physical and chemical factors, including the concentration and chemical structure of contaminant, physicochemical properties of soil, the content of biogenic salts, moisture content, oxygen and other terminal electron acceptor availability, organic compounds level, temperature and pH of soil. The rate and efficiency of the purification process of soil depends on the occurrence of adequately numerous and active microflora in the contaminated soil (Sobral et al., 2009; Zanaroli et al., 2010). Bioaugmentation involves the addition of microorganisms, indigenous or exogenous to the contaminated sites (Abu et al., 2016). A limiting factor in the use of microbial cultures in land treatment unit is that non-indigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels; and most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degraders if the land treatment unit is well managed (Silva-Castro et al., 2013; Cerqueira et al., 2014). Soil bioaugmentation is a solid phase process where specific microorganisms are added to the soil in order to enhance its biological activities. The seeded microorganisms are often developed through an enrichment process. This procedure results in the selection of the most efficient microorganisms that possess the necessary metabolic pathway and enzymatic

system for degradation of contaminants (Thompson et al., 2005; Sprocati et al., 2012). Soil bioaugmentation is most effective when the soil is not nutrient deficient, but the indigenous microbial population lacks the required metabolic activity. However, this technology has a limited capacity if the bioavailability of the contaminants, controlled by their desorption from soil, is the rate-limiting step in bioremediation (Laleh et al., 2003). Therefore, bioremediation protocols involving application of exogenous competent organisms as a supplement to those naturally present can improve the rate of recovery of polluted environments. Of course, the inoculation of diesel-contaminated soil with microbial consortia having high metabolic activity is essential in achieving effective bioremediation (Zanaroli et al., 2010). But despite the apparent simplicity of bio-augmentation, there have been many failures (Vogel and Walter, 2001; Wagner, 2003; Liu et al., 2016). Some of these failures have been attributable to harsh environmental conditions, pH and redox factors, the absence of key co-substrates (Thompson et al., 2005; Liu et al., 2016). Co-substrates such as surfactant and organic wastes (such as poultry waste, wheat straw) has shown to improve the bioremediation efficiency of diesel-contaminated soils (Soleimani et al., 2013; Laleh et al., 2016). Consequently, exploring different microbial species, their optimum degrading parameters, co-substrates or nutrient supplements with high efficiency to breakdown/degrade hydrocarbons, is of importance in the bioremediation of crude oil and its products. The objective of this study was to investigate the bioremediation of diesel-polluted soil using augmented bacteria and pig dung as nutrient supplement.

MATERIALS AND METHODS

Source of soil used

Unpolluted soil (7 kg) was collected from the Botanical Garden, University of Lagos, Lagos State, Nigeria.

Source of hydrocarbon used

The type of crude oil used is bony light from Shell Flow Station near Portharcourt (4°49`N 7°2`E), River State. The diesel oil used was bought from Total Filling Station in Ketu, Lagos (6°35`N 3°45`E).

Bioremediation protocols

Two kilograms of the soil contained in open tray, 13.5 x 8.5 x 4 cm (internal dimension) was contaminated with 200 g of diesel oil, to give approximately 10% (v/w) pollution. The contaminated soil in the tray was then inoculated with 200 mL of 8.8 x 10^{-3} cfu/ml of bacteria (*Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria*) capable of degrading hydrocarbon and supplemented with 200 g of pig dung (powder) thoroughly mixed and was subsequently designated A. The second tray contained 2 kg of soil sample contaminated with 200 mL of diesel oil only and labelled as B. The last tray C, which serves as the second control contain 2 kg of sterilized soil and 200 mL of diesel oil covered with foil paper to prevent contamination. To achive sufficient aeration, the content of the trays were mixed thoroughly every 3 days. Immediately after starting the experiment, and at intervals, 1 g of a polluted soil sample of each tray were taken to evaluate the bacterial population of the polluted soil sample. The collected samples were either analysed immediately or stored in a refrigerator at 4°C and later analyzed.

Determination of physiochemical parameters

The pH, moisture content, total phosphate, exchangeable bases (sodium, potassium, magnesium and calcium) and nitrogen were determined using methods described by AOAC, (2012).

Isolation and counting of organism from samples

Measured 9.0 mL of water in McCartney bottles were used as diluents for this purpose. One gram of the soil sample in each tray was weighed using weighing balance into McCartney bottle containing 9.0 ml of sterilized water and shaken vigorously. This was taken as 10^{-1} dilution measured using sterile pipette. An aliquot (1.0 ml) was then taken from this tube into a fresh tube with 9.0 ml sterile water to give 10^{-2} dilution. This process was continued until 10^{-10} dilution of the sample was obtained. Thereafter, 0.1 ml aliquot of 10th dilution was introduced into freshly prepared nutrient agar and spread thoroughly, using a hockey stick until the agar surface becomes dry. Plates were incubated at 30°C for 24 h. Bacteria population was monitored every three days. The resulting colonies were later sub cultured unto fresh plates by streaking along line of inoculation and gradually thinned out to obtain distinct and well separated colonies. The platinum loop was flamed after each streak or transfer.

Isolation of hydrocarbon utilizers

The isolation of hydrocarbon oil degraders was done by seeding the minimal salts agar medium with colonies isolated from the samples. Each was then inverted on to a Petri dish cover containing filter paper soaked with the tested hydrocarbon (soil sample + hydrocarbon). The hydrocarbon served as the major carbon source. Incubation was done at 28°C for 48 to 72 h. Each colony was then picked with a sterile aluminium loop, emulsified in distilled water and 0.1 ml aliquots plated unto the minimal salts agar plates to obtain distinct colonies. The isolated strains were maintained on nutrient agar medium, incubated for 24 h and kept at 4°C. The ability of these organisms to degrade the hydrocarbon was further tested by culturing on minimal salt broth with each hydrocarbon as sole carbon source. Each test-tube was filled with 9.0 ml minimal salt broth, 1% hydrocarbon to be tested, both autoclaved at 121°C for 15 min.

The isolates were then aseptically inoculated into the minimal salt broth and plugged with cotton wool to allow for aeration. These tubes were incubated at 30°C for 7 to 14 days with intermittent shaking to allow contact between the oil phase and the liquid phase, which contain the bacterial isolate. The amount of growth was observed comparatively with the control medium set-up, containing no bacterial inoculum (Amund et al., 1987).

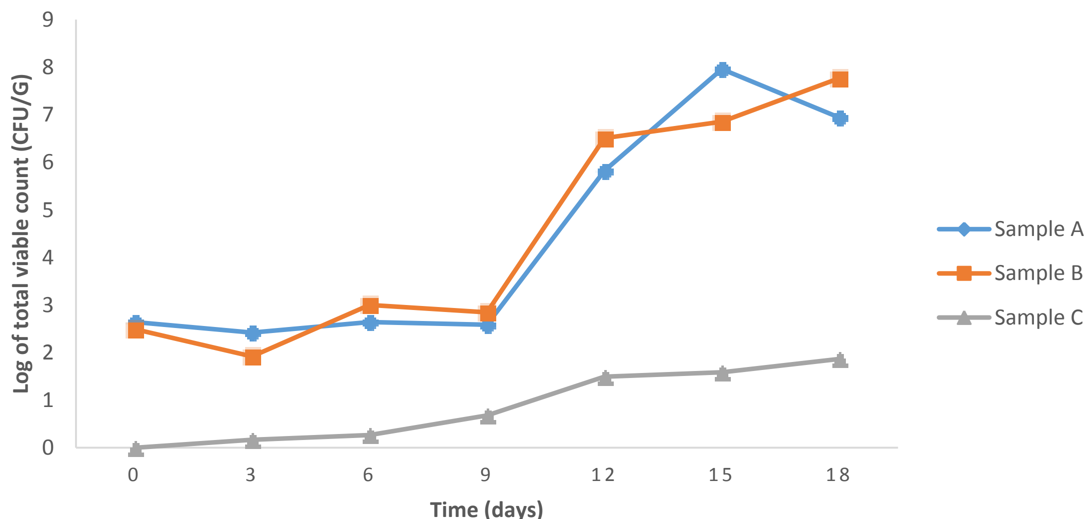

Figure 1. Growth profile of bacteria present in Trays A, B and C polluted with diesel oil.

Identification of the bacteria isolates

Bacterial isolates were prepared on agar plates and the characteristics of the colonies of the pure cultures were observed, recorded and used for their probable identification. Subsequently, biochemical tests were carried out on the bacterial isolates. These include: Gram's staining, motility, catalase test, oxidase test, gelatine hydrolysis, citrate utilization, indole production, methyl red test, acetylmeyhyl carbinol production (Voges Proskauer test), carbohydrate metabolism (hush and leifson's test), starch hydrolysis, acid-fast test, urease activity, nitrate test, acid and gas production from sugars.

Determination of residual oil concentration

The residual diesel concentration in the soils samples were determined according to Vallejo et al. (2001), 8 g of the soil were extracted using 20 ml hexane in a flask tilted with a cap. The residual diesel was analysed by 8200 auto sampler gas chromatography equipped with a 50 m fused silica open tube capillary column internal coated with crosslinked methyl silicon and flame ionization detection. The degradation percentage was determined using the following formula according to Bento et al. (2005).

Percentage degradation of diesel = {(Total diesel in Tray "C" – Total diesel in Tray "A/B")/Total diesel in Tray "C"}*100.

Tray A = soil + diesel + organisms + pig dung; Tray B = soil + diesel; Tray C = sterile soil + diesel.

RESULTS

The pH value of the soil sample was 6.9, while that of pig dung was 5.5. The moisture content of the soil was 12.9% while that of pig dung was 9.9%. The nutrient present in soil sample was lower as compared to that of the pig dung.

Substrate specificity test

The bacteria isolated from the soil sample were: *Pseudomonas* spp., *Pseudomonas cepacia, Micrococcus luetus, Bacillus subtilis* and *Bacillus cereus.* The ability of the bacteria to degrade hydrocarbons varies depending on the type of substrates available. Therefore, their ability to degrade the different substrates were observed by the presence of growth in the broth (Minimal Salt Broth + hydrocarbon been tested) (Table 2). All the isotate were unable to grow on hexane. LB1 and LB2 had a strong growth on n-dodecane and n-hexadecane. Only LB1, LB4 and LB5 were able to grow on paraffin. LB1 and LB5 had poor growth on xylene. LB1 and LB3 had moderate growth in phenol. All the isolates had little growth in kerosene and only LB3 and LB4 had poor growth in diesel.

Bacterial population

Figure 1 illustrates the different growth phases of the bacteria in each of the trays. Bacteria population in tray "A" reduces from days 15 to 18, an indication of nutrient exhaustion, while tray "B" and "C" still show potential to increase after day 18. This can be attributed to the presence of some residual diesel in the contaminated soil sample that can still be used by the bacteria in those trays.

Residual oil concentration

Tray "A" had a degradation of 73.82%, tray "B" had 40.95% and tray "C" had a degradation of 35.76%. Tray "A" shows the highest percentage followed by tray "B"

Table 1. Physiochemical parameters of soil and pig samples prior to bioremediation.

Parameter	Levels detected	
	Soil	Pig dung
pH	6.9	5.5
Moisture (%)	12.9	9.9
Sodium (Na) (%)	0.005	0.4
Potassium (K) (%)	0.002	0.06
Magnesium (Mg) (%)	0.04	0.6
Calcium (Ca) (%)	0.18	0.9
Nitrogen (N) (%)	0.02	1.2
Phosphorus (P) (%)	0.001	0.002

Table 2. Substrate specificity test of isolates on different carbon sources.

Substrates	LB1	LB2	LB3	LB4	LB5
Crude oil	+	++	-	+++	++
Xylene	+	-	-	-	+
Phenol	++	-	++	-	-
Engine oil	+	-	-	+	-
Diesel	-	-	+	+	-
Kerosene	+	+	+	+	+
Benzene	+	+	-	+	+
Cyclohexane	-	+	+	+	+
Paraffin	+	-	-	++	+
n- Decane	++	-	-	-	+
n-dodecane	+++	++	+	+	-
n-hexadecane	+++	+++	-	-	++
Hexane	-	-	-	-	-

No growth, + Poor growth, ++ Moderate growth, +++ Strong growth, LB1- *Pseudomonas* spp, LB2- *Micrococcus luetus,* LB3- *Bacillus subtilis,* LB4- *Bacillus cereus,* LB5- *Pseudomonas cepacia*

and this was due to bio augmentation and natural attenuation respectively of the polluted soil samples. The degradation obtained in Tray "C" can be attributed to volatilization of the diesel oil and probably later by invading bacteria.

DISCUSSION

Bioremediation of diesel-contaminated soil which involves the use of augmented bacteria and pig dung to reduce high levels of diesel to levels that can be harmless/safe and as a result, will minimize the subsequent damages caused to the environment. Bacteria either pure or mixed cultures used for the bioremediation of diesel-contaminated soils require nutrients, carbon and energy sources to grow and proliferate in harsh polluted environments (Lahel et al., 2016). Also, of importance is that, biodegradation/bioremediation rates of these microbes depend on the hydrocarbon composition and environmental conditions such as temperature, pH, moisture content, bioavailability of the pollutant, contamination levels and the presence of additional nutrients (such as pig dung in this research). Competition between indigenous and exogenous microorganisms for limited carbon sources, as well as antagonistic interactions and predation by protozoa and bacteriophage determines the final outcome of the bioremediation process (Franco et al., 2014; Fernandez et al., 2016; Lahel et al., 2016). The soil sample (soil sample before pollution) in this study was nutrient deficient (Table 1). The moisture content was also low and this is an important factor that can have an adverse effect on the metabolic activities of microorganisms during biodegradation of hydrocarbon (Van hammed et al., 2003; Thompson et al., 2005; Abu et al., 2016). The

Table 3. Residual oil concentration and % degradation of each sample after 18 days.

Tray	Oil concentration at day 0 (mg/g)	Oil concentration at day 18 (mg/g)	Degradation (%)
Tray A	246097.02	64439.55	73.82
Tray B	246097.02	145323.59	40.95
Tray C	246097.02	158083.07	35.76

Tray A = soil + diesel + organisms + pig dung, Tray B = soil + diesel, Tray C = sterile soil + diesel (control).

pH of the soil was 6.9, which was within the optimum range for microbial activities. It has been reported that such soil is ideal for hydrocarbon degrading microorganisms to be adapted in a bioaugmentation process (Lahel et al., 2016). The pig dung contains more nutrients (such as sodium, magnesium, calcium and nitrogen) as compared to that of the unpolluted soil sample. This is an additional nutrient source for the indigenous microorganisms and the inoculated bacteria. The presence of diesel in the soil samples had an adverse effect on the initial bacterial populations (Figure 1). However, some of the indigenous and the augmented bacteria that can be described as hydrocarbon utilizers (Table 2), soon adapted to the diesel-contaminated environment and utilized the diesel as a substrates for growth and this is the reason for increased bacterial population from the 9th day (Yakimov et al., 2007; Maduka and Okpokwasili, 2016). The bacterial activities in Tray "A" must have been boosted by additional nutrient from the pig dung and complimented by the inoculated crude oil degrading bacteria. These resulted in the rapid utilization of the diesel substrate (Figure 1). The bacterial population in tray "A" shows an initial decrease from day 0 to day 3, which is due to the toxicity of the diesel oil and a steady adapting lag phase from day 3 till day 9. The exponential phase is observed from day 9 to 15, having day 15 as peak growth. The observed reduction in the population density from days 15 to 18 is due to the exhaustion of nutrients (diesel oil depletion). The initial reduction in the population density from day 0 to day 3 in tray "B" can also be attributed to the toxic effect of diesel on the microorganisms present in the soil sample. The relative increase from days 3 to 9, then steady continuous increase in growth showing availability of more nutrient that can be utilized by the microorganisms' presence. Due to the use of sterile soil in Tray "C", the bacterial population was low at the early days and later increases steadily from day 6 till day 18 (still lower bacterial population as compared to those of Trays "A" and "B"). Table 3 shows the extent of diesel degradation/mineralization during the bioremediation of diesel-contaminated soil by bioaugmentation process supplemented with pig dung. Mineralization of diesel was found to be highest (73.82%) in tray "A" and lowest in tray "C" (35.76%). Bioaugmentation using the consortia of *B. coagulans, C. koseri* and *S. ficaria* supplemented with pig dung was effective in degradation of diesel-contaminated soil made up of 200 mL of diesel in 2 kg of soil approximately 10% (v/w) contamination. Nevertheless, this was not a complete removal of the diesel contaminant. Complete removal or higher degradation percentage will be preferred because the residue might be recalcitrant by-products from the initial diesel biodegradation which could lead to bioconcentration, bioaccumulation and biomagnification in a real life scenario. The by-products could also have toxic effect on soil organisms (Agnieszka and Zofia, 2010; Hou et al., 2013; Wu et al., 2016; Lahel et al., 2016). Microbial species capable of degrading all the constituents of crude oil/crude oil products are limited in number. Probably, because the inherent ability of each strain/species to degrade one or more hydrocarbon compound is individually confined to them. Hence, efficient degradation of hydrocarbon requires a consortium composed of various microbial strains (Yousseria et al., 2016). The consortium approach improves biodegradation ability. Microbial mixed cultures or consortia have a higher ability to adapt to stress conditions and therefore show increased microbial survival. In addition, they can increase the number of catabolic pathways available for diesel biodegradation and can easily prevent or reduce the accumulation of recalcitrant/toxic compounds from microbial degradation (Briceno et al., 2016). Therefore, increase in the concentration/dose of augmented bacteria could improve the amount of diesel mineralized. Higher microbial dose is believed to boost the adaptability and assimilation capacity of microbial population to the newly introduced soil, which in turn results in higher mineralization efficiency (Trindade et al., 2002; Lahel et al., 2016). The soil sample in Tray "B", which underwent natural attenuation, resulted in residual diesel of 59% meaning that 41% of the diesel was lost through the activities of indigenous organisms who had utilized the diesel (Bento et al., 2005; Silva et al., 2015). *Pseudomonas* spp., and *Bacillus* spp. which were some of the indigenous bacteria present in the soil sample, have actually been implicated many times in hydrocarbon degradation (Perfumo et al., 2007; Alfreda and Ekene, 2012; Collado et al., 2013). The indigenous organisms utilizing the diesel could take longer time. This will not be in the interest of maintaining healthy environment without harm to the organisms in that environment; hence, the need for effective method such as bioaugmentation and biostimulation. The impact

of hydrocarbons on microorganisms may not be directly related to their toxicity. Several researchers have reported destruction of inorganic nutrient sources that are essential for microbial growth and catabolic activities due to the ability of hydrocarbons to react and form complexes with nitrates, sulphates and phosphates, thus making them unavailable to oil degrading organisms (Andrew and Jackson, 1996; Abu et al., 2016). The activities in tray "C", was also probably influenced by the soil sorption property. Judging from the conditions in the tray and the bacterial load, it can be said that apart from the microbial activities, some other factors (soil sorption, adsorption, desorption and volatilization) could have influenced diesel concentration in soil sample in Tray "C". The contaminated soil samples in the other two trays (tray "A" and " B") could also have been influenced by same factors. Soil potential to sorb a certain amount of diesel has been documented, especially the clay and humus compounds present in the soil (Lahel et al., 2016). Falciglia et al. (2011), also noted that adsorption and desorption efficiency of diesel is usually affected by the type of soil texture.

Conclusion

The effect of augmented *B. coagulans*, *C. koseri* and *S. ficaria* in biodegradation of diesel-contaminated soil was more effective than natural attenuation in this present study. Their effectiveness can be improved by optimizing bioremediation parameters such as pH, temperature, substrate concentration and moisture content. Cell immobilization technique could as well enhance the degradation activities of these bacteria consortia. Also, the bacterial species isolated in this work had potential for hydrocarbon degradation, with further research, they can be used in the degradation of hydrocarbons. Since they do not occur as pure cultures in nature, the consortia of these organisms could show greater biodegrading abilities.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This material is based on work financially supported by Costech Canada Inc.

REFERENCES

Abu L, Abaynesh BF, Natalia S, Manisha S, M.Estefania S, Shishir KB, Eldon RR, Hung-suck P (2016). Effect of process parameters on the bioremediation of diesel contaminated soil by mixed microbial consortia. Int. Biodeterioration and Biodegr. 113:375-385.

Adams P, Jackson P (1996). Bioremediation of oil spills, theory and practice. Proceedings of International Seminar on Petroleum Industry and the Nigeria Environment, Port Harcourt, Nigeria; 1:30-42.

Agnieszka M, Zofia P-S (2010). Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiological Research, 165(5):363-375.

Alfreda ON, Ekene GO (2012). Bioremediation of crude oil polluted soil using bacteria and poultry manure monitored through soybean productivity. Polish J. Environ. l Stud. , 21(1):171-176.

Amund OO, Adebowalre AA, Ugoji EO (1987). Occurrence and characteristics of hydrocarbon-utilizing bacteria in Nigerian soils contaminated with spent motor oil. Indian J. Microbiol. 27:63-67.

AOAC (2012). Official Method of Analysis: Association of Analytical Chemists. 19th Edition, AOAC, Washington DC, pp. 121-130.

Bento FM, Camargo FAO, Okeke BC, Frankenberger WT (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96:1049-1055.

Bossert I, Compeau G (1995). Cleanup of Petroluem hydrocarbon contaminated soil. Microbial Transformation and degradation of toxic chemicals, 77-125.

Bragg JR (1994). Effectiveness of Bioremediation for the Exxon Valdex Oil Spill. Nature J. 368:413-418.

Briceno G, Schalchli H, Mutis A, Benimeli CS, Palma G, Tortella GR, Diez MC (2016). Use of pure and mixed culture of diazinon-degrading Streptomyces to remove other organophosphorus pesticides. Int. Biodeterior. Biodegr. 114:193-201.

Cerqueira VS, Peralba MDCR, Camargo FAO, Bento FM (2014). Comparison of bioremediation strategies for soil impacted with petrochemical oily sludge. Int. Biodeterior. Biodegr. 95:338-345.

Chandran P (2011). Microbial degradation of petroleum hydrocarbon contaminants: An overview. Biotechnol. Res. Int. pp. 1-13.

Chemlal A, Abdi N, Lounici H, Drouiche N, Pauss A, Mameri N (2013). Modeling qualitative study of diesel biodegradation using biopile process in sandy soil. Int. Biodeterior. Biodegr. 78:43-48.

Collado N, Buttiglieri G, Kolvenbach BA, Comas J, Corvini PF, Rodriguez-Roda I (2013). Exploring the potential of applying proteomics for tracking bisphenol A and nonylphenol degradation in activated sludge, Chemosphere, 90:2309-2314.

Das N, Chandran P (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. Res. Int. 11:11-13.

Erin NK, David WS, Peter VH, Jeffrey WS, Roseanna R, Charlene CN (2010). Oil sands development contributes elements toxic at low concentrations to the Athabasca River and its tributaries. Proceedings of the National Academic of Sciences of the United States of America, 107(37):16178-16183.

Esin (Eraydin) E, Ayten K (2011). Bioremediation of Crude Oil Polluted Soils. Asian J. Biotechnol. 3:206-213.

Falciglia PP, Giustra MG, Vagliasindi FGA (2011). Low-temperature thermal desorption of diesel polluted soil: in fl uence of temperature and soil texture on contaminant removal kinetics. J. Hazard. Mater. 185:392-400.

Fernandez EL, Merlo EM, Mayor LR, Camacho JV (2016). Kinetic modelling of a diesel-polluted clayey soil bioremediation process. Science of the Total Environment, pp. 557-558.

Franco MG, Correa SM, Marques M, Perez DV (2014). Emission of volatile organic compounds and greenhouse gases from the anaerobic bioremediation of soils contaminated with diesel. Water, Air and Soil Pollution, 225(2):1-9.

Hou D, Shen X, Luo Q, He Y, Wang Q, Liu Q (2013). Enhancement of the diesel oil degradation ability of a marine bacterial strain by immobilization on a novel compound carrier material. Marine Pollution Bulletin, 67:146-151.

Ibrahim ML, Ijah UJ, Manga SB, Rabah, AB (2008). Occurence of Hydrocarbon Utilizing Bacteria in the Rhizophere of Eucalyptus Camaldulasis, Lablab Purpefeus and Moriga Oleifera. Intl. J. pure Appl. Sci. Technol. 2(3):21-26.

Jain DK, Lee H, Trevors JT (1992). Effect of addition of Pseudomonas aeruginosa UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil; tetradecane degradation, hexadecane degradation and pristane degradation, and soil decontamination by

bacterial cells or surfactant. J. Ind. Microbiol. 10:87.
Lahel A, Fanta AB, Sergienko N, Shakya M, Lopez ME, Behera SK, Rene ER, Park H (2016). Effect of process parameters on the bioremediation of diesel contaminated soil by mixed microbial consortia. Int. Biodeterior. Biodegr. 113:375-385.
Laleh Y, Sylvie R, Ruxandra C, Manon S, Geoffery S, Adriana P, Gervais L (2003). Enhanced biodegradation of petroleum hydrocarbons in contaminanted soil. Bioremediat. J. 7(1):37-51.
Liu PWG, Yang D, Tang J, Hsu H, Chen C, Lin I (2016). Development of a cell immobilization technique with polyvinyl alcohol for diesel remediation in seawater. Int. Biodeterior. Biodegr. 113:397-407.
Maduka CM, Okpokwasili GC (2016). Microbial growth in brake fluid. Int. Biodeterior. Biodegr. 114:31-38.
Mielke JE (1990). Oil in the Ocean. The Short Term and Long Term Impacts of a Spill. Library of Congress, Congressional Research Service.
Nwachukwu SCU (2000). Modelling the recovery rate of a tropical soil ecosystem polluted with crude petroluem, using *Pseudomonas* spp. As the test organisms. Int. J. Environ. Education inform. 16(1):55-62.
Perfumo A, Banat IM, Marchant R, Vezzulli L (2007). Thermally Enchanced Approaches for Bioremediation of Hydrocarbon-Contaminated soil. Chemosphere. 66 (1):179-184.
Roy AS, Baruah R, Borah M, Singh AK, Deka Boruah HP, Saikia N, Deka M, Dutta N, Chandra BT (2014). Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. Int. Biodeterior. Biodegr. 94:79-89.
Rui L, Rajendrasinh NJ, Qixing Z, Zhe L (2012). Treatment and Remediation of Petroleum-Contaminated Soils Using Selective Ornamental Plants. Environ. Eng. Sci. 29(6):494-501.
Silva DSP, Cavalcanti DL, Melo EJV, Santos PNF, Luz ELP, Gusm~ao NB, Sousa QMDFV (2015). Bio-removal of diesel oil through a microbial con- sortium isolated from a polluted environment. *International Biodeterioration and Biodegradation*, 97, 85 - 89.
Silva-Castro GA, Rodelas B, Perucha C, Laguna J, Gonzalez-Lopez J, Calvo C (2013). Bioremediation of diesel-polluted soil using biostimulation as post- treatment after oxidation with Fenton-like reagents: assays in a pilot plant. Sci. Total Environ. 445:347-355.
Sobral GS, Millioli ELC, Servulo, De Carvalho D (2009). Bioremediation of crude oil-bearing soil: evaluating the effect of rhamnolipid addition to soil toxicity and to crude oil biodegradation efficiency. Global NEST J. 11(2):181-188.
Soleimani M, Farhoudi M, Christensen JH (2013). Chemometric assessment of enhanced bioremediation of oil contaminated soils. J. Hazard. Mater. 254:372-381.
Sprocati AR, Alisi C, Tasso F, Marconi P, Sciullo A, Pinto V, Chiavarini S, Ubaldi C, Cremisini C (2012). Effectiveness of a microbial formula, as a bio- augmentation agent, tailored for bioremediation of diesel oil and heavy metal co-contaminated soil. Process Biochem. 47:1649-1655.
Thompson IP, Vander Gast CJ, Ciric L, Singer AC (2005). Bioaugmentation for bioremediation: the challenge of strain selection. Environ. Microbiol. 7:909-915.
Trindade PVO, Sobral LG, Rizzo ACL, Leite SGF, Lemos JLS, Millioli VS, Soriano AU (2002). Evaluation of the biostimulation and bioaugmentation techniques in the bioremediation process of petroleum hydrocarbons contaminated soil. In: Proceedings of the 9th Annual International Petroleum Environmental Conference October, 22-25.
Vallejo A, Diez JA, Lopez-Valdivia LM, Gasco A, Jimenez C (2001). Nitrous oxide emission and denitrification N-losses from soils treated with isobutylidendiurea and urea plus dicyandiamide. Biol. Fertil. Soils, 34:248-257.
Van Hammed, JD, Singh A, Ward OP (2003). Recent Advances in Petroleum Microbiology. Microbiol. Mol. Biol. Rev. 67(4):503-549.
Vogel TM, Walter MV (2001). Bioaugmentation. In Hurst CJ, Crawford RL, Knudsen GR, Mclnerney MJ, Stetzenbach LD (eds) Manual of environmental microbiology, ASM Press Washington DC, pp. 952-959.
Wagner DI (2003). Microbial inoculants: snake oil or? In: Head IM, Singleton I, Milner MG (eds) Bioremediation: a critical review, Horizon scientific Press, Norfolk, pp. 259-289.
Wang Z, Fingas M, Sergy (1994). Study of 22 year old Arrow oil samples using biomaker compounds by GC/MS. Environ. Sci. Technol. 28:1733 -1746.
Wu M, Dick WA, Li W, Wang X, Yang Q, Wang T, Xu L, Zhang M, Chen L (2016). Bioaugmentation and biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated soil. Int. Biodeterior. Biodegr. 107:158-164.
Yakimov MM, Timmis KN, Golyshin PN (2007). Obligate oil-degrading marine bacteria. Curr. Opin. Biotechnol. 18(3):257-266.
Yousseria MHS, Wafaa AAE, Tarek MM, Laila AF, Ahmed E (2016). Potential biodegradation of crude petroleum oil by newly isolated halotolerant microbial strains from polluted Red Sea area. Mar. Pollut. Bullet. 111(1-2):435-442.
Zanaroli G, Toro SD, Todaro D, Varese GC, Bertolotto A, Fava F (2010). Char- acterization of two diesel fuel degrading microbial consortia enriched from a non-acclimated, complex source of microorganisms. Microb. Cell Fact. 9:1459-1475.

academicJournals

Vol. 10(39), pp. 1645-1653, 21 October, 2016
DOI: 10.5897/AJMR2016.8254
Article Number: BEC23A961246
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* isolates from Egyptian hospitals

Marwa M. Raafat[1], Marwa Ali-Tammam[1] and Amal E. Ali[1,2]*

[1]Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences and Pharmaceutical Industries. Future University, 12311 Cairo, Egypt.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Egypt.

Received 4 August 2016, Accepted 11 October, 2016.

***Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen and a leading cause of hospital-acquired infections. Characterization of the isolates from different infection sites might help to control infections caused by the pathogen. The aim of the present work is to characterize *P. aeruginosa* isolates recovered from different clinical specimens at two hospitals in Cairo with regard to their antibiogram, genotypes and virulence factors. The highest antimicrobial resistance pattern was exhibited by isolates from sputum. Resistance rate recorded for sputum samples to different in- use antibiotics was 80, 80-100, 36, 54 and 54% for Penicillins, Cephems, Carbapenems, Aminoglycosides and Fluoroquinolones, respectively. Phenotypic detection of virulence factors in *P. aeruginosa* isolates included detection of protease, lecithinase, DNase, hemolysin and pyocyanin revealed that, each isolate had at least one virulence factor. Protease and lecithinase were the most commonly detected, where 68 and 66% of the isolates showed positive protease and lecithinase activities respectively. Random amplified polymorphic DNA (RAPD) genotyping using 2 random primers revealed 22 and 14 different genetic profiles. Phylogenetic trees based on genetic distances showed 3 clusters with obvious similarity between some isolates, indicating common sources of infection. No association could be found between clustering pattern of the isolates, their antibiogram and virulence.**

Key words: *Pseudomonas aeruginosa*, antibiogram, genotypes, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR).

INTRODUCTION

Pseudomonas aeruginosa is a Gram negative highly opportunistic pathogenic bacteria commonly isolated from hospitalized patients. It is a leading cause of nosocomial infections particularly in immunocompromised patients

*Corresponding author. E-mail: amal.ali@pharma.cu.edu.eg. Tel: 2-0100-8551561, 2-02-26186100.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

Table 1. Number of *P. aeruginosa* isolates recovered from different clinical sources.

Source	Number of isolates (percentage)	Hospital/number of isolates
Urine	12 (26.7)	Al- AzharUniversity (5); Kasr Al Ainy (7)
Wound	8 (17.8)	Al- AzharUniversity (8); Kasr Al Ainy (0)
Sputum	11(24.4)	Al- AzharUniversity (5); Kasr Al Ainy (6)
Abscess	11(24.4)	Al-AzharUniversity (9); Kasr Al Ainy (2)
Stool	3 (6.7)	Al-AzharUniversity (2); Kasr Al Ainy (1)

(Zenone and Souillet, 1996). *P. aeruginosa* accounts for 10 to 22.5% of hospital acquired infections in Asia-Pacific, Europe, Latin America, United States, Canada and Italy (Gales et al., 2001; Simonetti et al., 2013). It causes infections such as bacteraemia, urinary tract infections, lung infections, cystic fibrosis, wound infections especially of thermal burns, surgical wound infections, and otitis media (Pollak, 1998; Schaechter et al., 2009).

It is widely known that *P. aeruginosa* infections cause significant morbidity and mortality due to the organism ability to adapt easily to environmental changes, to develop resistance to antibiotics and to produce a wide variety of virulence factors (Van Delden and Iglewski, 1998), such as the formation of pyocyanin, hemolysin, gelatinase and biofilm which lead to tissue damage and protect *P. aeruginosa* against the recognition of the immune system and action of antibiotics (Cevahir et al., 2008; Ciofu et al., 2008; Todar, 2009); in addition *P. aeruginosa* is capable of producing other virulence factors as lipase, lecithinase, DNase and protease.

P. aeruginosa has different mechanisms of resistance, which generates multi-drug resistant isolates or pan-resistant isolates (Jácome et al., 2012). Several mechanisms of resistance which the pathogen develops include multidrug efflux pump, production of *β*-lactamases and aminoglycoside modifying enzymes (Mahmoud et al., 2013) which reflect the increasing rate of multidrug resistant strains worldwide causing a serious problem in hospital settings (Lim et al., 2009).

The molecular studies aimed to determine diversity among the pathogen isolates, polymorphism of certain of its genes and also genetic comparison of *P. aeruginosa* isolates from different hosts and environments (Kiewitz and Tummler, 2000; Martin et al., 1999; Rumiy et al., 2001; Onasanya et al., 2010).

One of the molecular techniques widely used in these studies include RAPD-PCR, a method based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams et al., 1990).The main advantage of RAPD-PCR over other molecular genotyping techniques is its simplicity, low cost and its ability to generate a large number of genetic markers without the requirement for cloning, sequencing or any other form of molecular characterization of the genome of the species (Kumar and Gurusubramanian, 2011).

The investigation of genotypes of the *P. aeruginosa* isolates, characterization of their antibiotic resistance pattern and virulence factors might be useful to identify the clonal relationship between isolates from different clinical specimens and to control infections caused by the pathogen.

Therefore, the aim of this study is to evaluate the antimicrobial resistance pattern, genetic diversity and virulence factors of strains of *P. aeruginosa* isolated from different sources of infections at two Egyptian hospitals.

MATERIALS AND METHODS

Specimen collection

A total of 45 *P. aeruginosa* isolates were obtained from the bacteriology laboratory of Al- Azhar University and Kasr Al Ainy Egyptian hospitals. Isolates were recovered from urine, wound, stool, abscess and sputum specimens as shown in Table 1. Isolates were identified according to the standard microbiological tests using gram staining, cetrimide agar (Oxoid, UK), Pseudomonas P (Difco, USA), Pseudomonas F (Difco, USA), Oxidase test and API 20NE (API BioMérieux, France).

Antibiotic susceptibility testing

Susceptibility test was performed according to Kirby- Bauer disc diffusion test (CLSI 2011) using the following antibiotic discs (Oxoid, UK): Amoxicillin/clavulanic Acid (AMC 30 µg),Amikacin (AK 30 µg), Ciprofloxacin (CIP, 5 µg), Ceftriaxone (CRO 30 µg), Trimethoprim sulfamethoxazole (SXT), Cefotaxime (CTX 30 µg) , Imipenem (IPM 10 µg), Meropenem (MEM 10 µg), Chloramphenicol (C 30 µg), and Tetracycline (TE 30 µg). Inhibition zone was recorded in mm. The susceptibility pattern was determined using the CLSI interpretation chart as susceptible (S), intermediate (I) and resistant (R).

Phenotypic detection of virulence factors

Determination of enzymatic activities

Enzymatic activities were evaluated by spot inoculation of an overnight culture of the organisms in various media: Skim milk agar for the protease activity, trypticase soy agar (TSA) supplemented with egg yolk for the lecithinase activity, DNase agar for the DNase activity (Matar et al., 2005). Plates were incubated for 24 or 48 h at

37°C. A positive result around the inoculum spot is indicated by a clear halo for protease activity, white precipitate for lecithinase activity and clear zone after adding 1 N HCl for DNase activity.

Test for pyocyanin

Test was performed according to the method of Huerta et al. (2008). Cultures were grown in Luria broth at 37°C at 120 rpm for 16 to 18 h. Cultures were centrifuged at 10,000 *g* at 4°C for 15 min. Cell free supernatants and cell pellets were separated. 3 ml of culture supernatant was mixed with 1.2 ml of chloroform. Mixture was incubated for 30 min at room temperature. Absorbance of chloroform layer was measured at 690 nm.

Production of hemolysin

Hemolysin production was assayed by growing the isolate overnight for 16 h in nutrient broth and spot inoculation of 10 µl onto sheep blood agar. Incubation done was at 37°C overnight. Hemolysin production was verified by the presence of clear haemolytic halo around the spot (Santo et al., 2006).

RAPD genotyping

Total DNA from *P. aeruginosa* isolates was obtained by suspending 1 to 5 colonies in 100 µl of nuclease free water. Suspension was heated at 99°C for 10 min, and then cooled on ice. After centrifugation at 13000 rpm for 5 min, 5 µl of supernatant was used as template in 25 µl PCR reaction (Nunes et al., 1999). Amplification was performed using primer 208 (5 'ACGGCCGACC 3') (Campbell et al., 2000), or primer 1281 (5 'AACGCGCAAC 3') (Akopyanz et al., 1992). RAPD PCR mix (25 µl) consisted of 10 x reaction buffer, 3 mM $MgCl_2$, 200 µm dNTP, 20 pm primer and 2 U Taq DNA polymerase, 5 µl DNA template.

Thermal cycler program was as follows: Initial denaturation of 5 min at 94ºC, followed by (I) 4 cycles with each cycle consisting of 5 min at 94ºC, 5 min at 36ºC and 5 min at 72ºC. (II) 30 cycles with each cycle consisting of 1 min at 94ºC, 1 min at 36ºC and 2 min at 72ºC and followed by a final extension step at 72ºC for 10 min. RAPD products were then separated by electrophoresis in 1% agarose gel at 80 v for 1 h. The RAPD fingerprints were analysed both by naked eye and by computer with Gel Compar II software with 1 Kb DNA ladder. The bands for each strain, were scored as absent (0) or present (1) in order to construct a genotype dendrogram. The similarities between fingerprints were determined by construction of a similarity matrix using the Jaccard coefficient and a dendrogram generated using unweighted pair group method with an average linkage (UPGMA) algorithm. Primer efficiency was calculated for each primer by using the following equation as described by Graham and McNicol (1995):

Primer efficiency = Total No. of bands produced by all primers / No. of total bands of primer *100.

RESULTS

Among the forty five *P. aeruginosa* isolates, 26.7% were recovered from urine, 24.4% from both sputum and abscess, 17.8% from wound and 6.7% from stool (Table 1). Isolates were characterized with regard to antimicrobial susceptibility profile, virulence factors and RAPD typing.

Antimicrobial profile

In this study, antimicrobial susceptibility profiles to 10 antibiotics, representing eight different classes, showed high rate of antibiotic resistance. It was observed that the percentage resistance to Ceftriaxone, Trimethoprim Sulfamethoxazole, amoxicillin/clavulanic acid, cefotaxime, meropenem, imipenem, amikacin, chloramphenicol, tetracycline, and ciprofloxacin was 95, 95, 84, 82, 22, 15%, 17, 48, 35 and 20%, respectively (Figure 1).

Virulence factors

Phenotypic detection of virulence factors in *P. aeruginosa* isolates included protease, lecithinase, DNase, hemolysin and pyocyanin. Protease and lecithinase activities were the most commonly detected virulence factors, where 68 and 66% of the isolates showed positive results for protease and lecithinase activities respectively. While only, 37, 22 and 13% of the isolates were positive for DNase, hemolysin and pyocyanin, respectively (Figure 3).

Recorded virulence factors exhibited variation according to the isolation source. The highest protease production was observed in isolates from sputum, while isolates from abscess and urine showed the highest lecithinase and DNase production respectively (Figure 4).

RAPD profile

Genetic analysis of 45 *P. aeruginosa* isolates revealed 22, 14 different profiles using primers 208 and 1281, respectively. Fingerprints consisted of 1 to 7 amplification bands ranging in size from 100 to 3 Kb. Some isolates showed the same profile bands. Figure 5 shows a representative banding profile of *P. aeruginosa* isolates following RAPD –PCR.

The dendograms created by UPGMA using primer 208 and primer 1281 are shown in Figures 6 and 7, respectively.

Three major clusters A, B and C are identified in both dendograms. Each cluster contained one or two groups which are further subdivided into subgroups. Sixty one-percent of isolates were placed in cluster C for dendogram created using primer 208 and 70% of the isolates were grouped in cluster B for dendogram created using primer 1281, while cluster A and cluster C contained only one isolate and three isolates in dendograms created using primer 208 and 1281, respectively. Some isolates were placed in the same subgroups with genetic

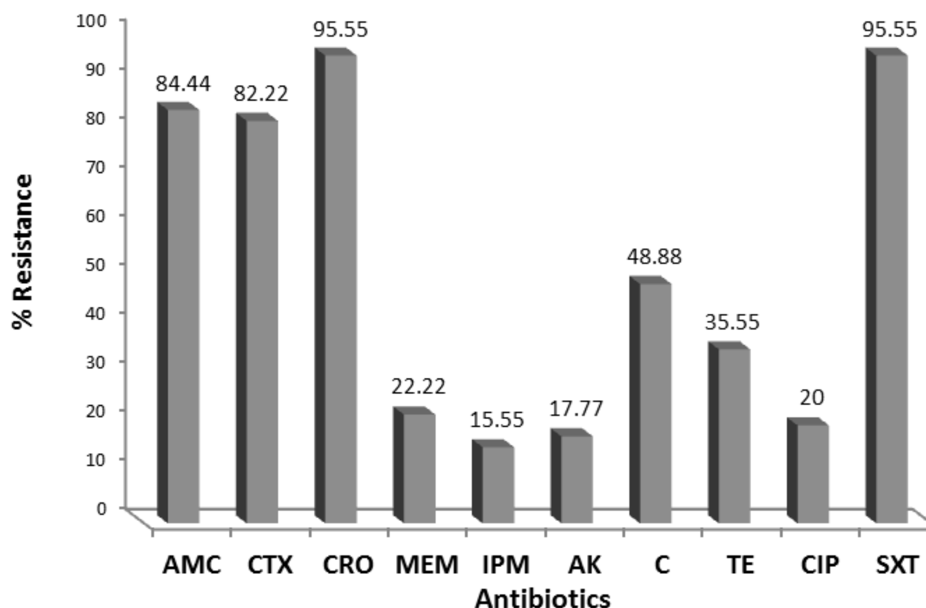

Figure 1. Antibiotic susceptibility profile for *P. aeruginosa* isolates.

distance of 0, indicating genetic similarity between them. Genetic distance ranged from 0 to 0.5 and from 0 to 0.25 for dendograms created using primer 208 and 1281, respectively.

DISCUSSION

In this study, we characterized and typed *P. aeruginosa* strains isolated from different clinical sources from Egyptian hospitals according to their antibiogram, genetic fingerprint and virulence factors. The antibiotics were selected to represent different classes. High rate of antibiotic resistance was observed compared to other studies of Ali and Balkhy in Gulf Cooperation Council countries (2012), Yayan et al. (2015) in Germany, and Bonfiglio et al. (1998) in Italy. These results might be justified by the extensive usage of antibiotics in Egyptian hospitals. Different resistance pattern were shown, where the most common pattern exhibited by 53% of the isolates was Amoxicillin/ clavulanic acid (R), Cefotaxime (R), Ceftriaxone (R), Trimethoprim sulfamethoxazole (R), Amikacin (S), Imipenem (S), Meropenem (S), and Ciprofloxacin (S). While the least common pattern shown by 13% was Amoxicillin/clavulanic acid (R), Cefotaxime (R), Ceftriaxone (R), Amikacin (R), Imipenem (R), Meropenem (R), Trimethoprim sulfamethoxazole (R), Ciprofloxacin (R), Tetracycline (R) and Chloramphenicol (S). Resistance rate recorded for sputum samples to different in- use antibiotics was 80, 80-100, 36, 54 and 54% for Penicillins, Cephems, Carbapenems, Aminoglycosides and Fluoroquinolones respectively, indicating different mechanism of resistance used by these isolates. Resistance rate of sputum samples was higher than those reported by Fatima et al. (2012) in Pakistan, who studied the susceptibility profile of *P. aeruginosa* isolates from patients with lower respiratory tract infection.

It is well known that, *P. aeruginosa* depends upon extracellular enzymes and toxins that break down physical barriers and damage host cells to invade tissues and overcome host immune defence (Cevahir et al., 2008). Among the enzymes and proteins involved in invasion are proteases, lecithinase, hemolysins and pyocianins pigments. Phenotypic detection of extracellular virulence factors revealed the presence of the tested virulence factors, including lecithinase, protease, pyocianin, hemolysin and DNase in variable percentages, where the most prevalent were lecithinase and protease. In this study, the results were in agreement with a study of Mashhadani (2004) in Iraq who reported that *P. aeruginosa* isolates from different clinical sources were positive for protease production (100%). In this study, the recorded DNase activity was higher in urine isolates. The results disagreed with Holban et al. (2013) who reported the absence of DNase in all tested UTI isolates and who justified that by the absence of viscous secretions and neutrophils extracellular traps in UTI infections due to its physiology which are the important stresses for DNase production. In our study, urine and sputum isolates were the most virulent strains expressing all tested virulence markers. This finding was also reported by Holban et al. (2013), that *P. aeruginosa* isolates from blood and respiratory tract isolates were the most virulent and was exhibiting the entire spectrum of

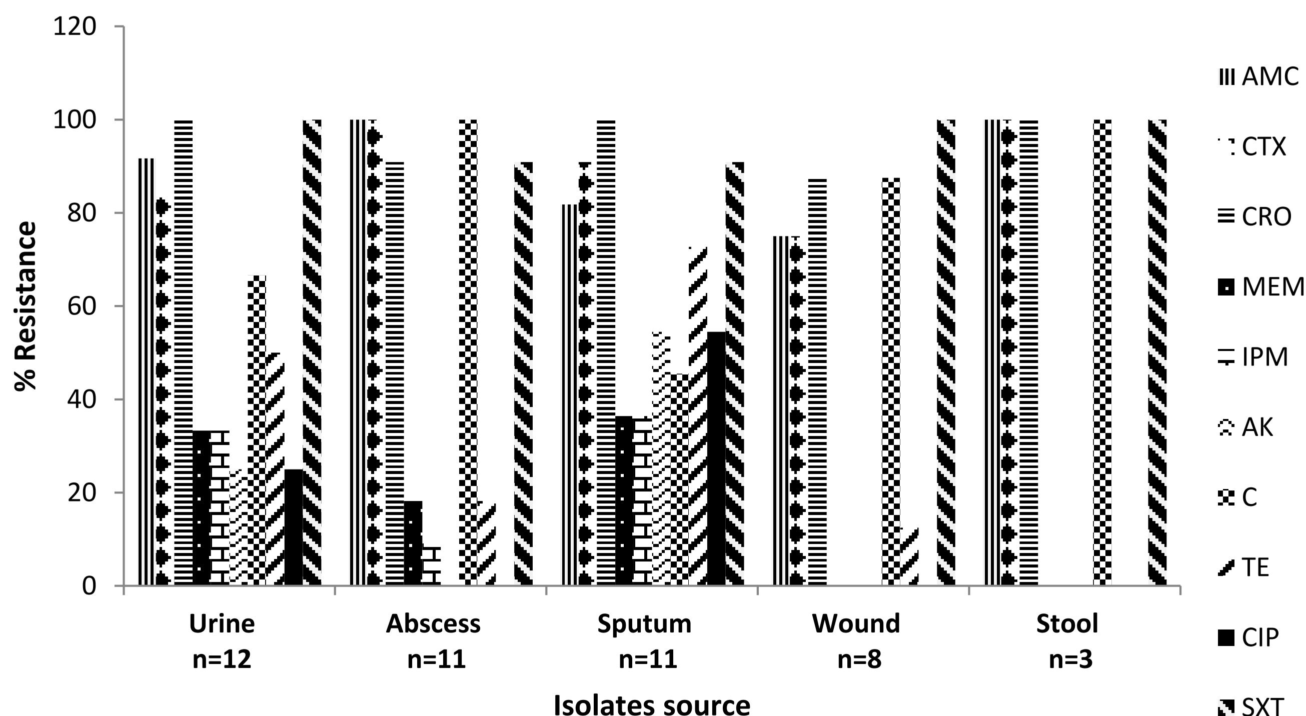

Figure 2. Antibiotics susceptibility profile of *P. aeruginosa* isolates in relation to clinical sources.

tested virulence factors. Variation of expressed virulence factors by *P. aeruginosa* isolates could be justified by the difference in signalling molecules present in different anatomical sites within the host and that could affect bacterial modulation of virulence factors for better persistence (Hughes and Sperandio, 2008).

Analysing the results of RAPD genotyping revealed that, the higher number of RAPD pattern was shown with primer 208 (22 patterns) compared with primer 1281 (14 patterns). Also**,** primer 208 showed 13 bands, compared to 9 bands for primer 1281. No monomorphic bands were shown for each primer. Primer 208 was more efficient (59%), whereas efficiency of primer 1281 was 40%. The efficiency of the primer reflects the availability of sequences complementary to the primer in the genome that allows base pairing between the primer and genomic DNA (Karp and Edward, 1997). These results were in agreement with the suggestion of Fristch et al. (1993) who reported that high efficiency of the primer is always correlating with the GC content of the primer.

Six isolates did not show amplification bands with primer 208, while primer 1281 failed to amplify DNA of 18 isolates, three of which did not show amplification products with both primers and are referred as untypeable. In that context, Menon et al. (2003) found one untypeable strain among 15 *P. aeruginosa* strains recovered from endophthalmitis cases. In addition, Zulkifli et al. (2009) who studied the diversity of *Vibrio parahaemolyticus* isolated from cockles in Indonesia, reported that, certain isolates failed to produce any products with the primers used.

Although, isolate number 6, 23 27, 40 38 and 46 were not discriminated in the dendogram using primer 208, they were discriminated in the dendogram using primer 1281, but were placed in sub-groups of higher similarities, which demonstrates the importance of using more than one primer in RAPD typing. In the dendogram using primer 208, isolate number: 40, 27, 26, 24, 23 and 6 from different isolation sites were from the same hospital, and were placed in the same sub-groups which indicate common sources of infections in hospitals environment, regardless of the isolation source and virulence. On the other hand, the dendogram using primer 1281, isolates grouped in cluster B were from the same hospital, except isolate number 45 and 44, which reinforces the observation of the common source of infection within the same hospital.

Differences in antibiotic resistance profile in tested strains were observed according to the isolation source. Isolates from sputum exhibited the highest resistance percentages to tested antibiotics, followed by isolates from urine (Figure 2).

Conclusion

This study shows a high rate of antibiotic resistance among *P. aeruginosa* isolates from different clinical

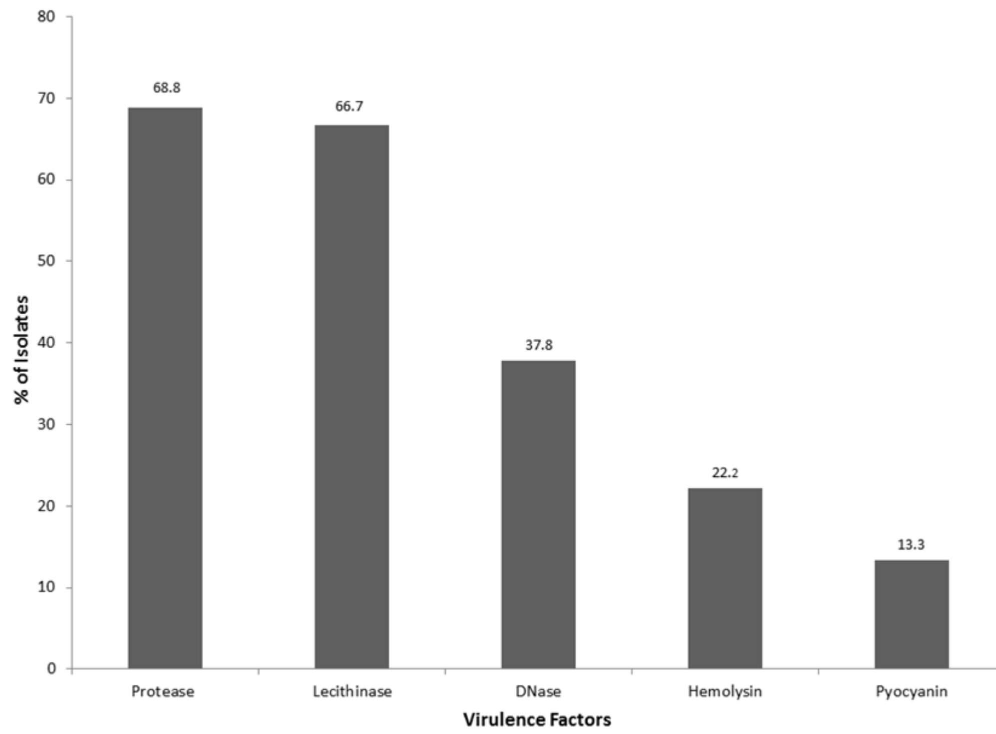

Figure 3. Virulence factors of *P. aeruginosa* isolates.

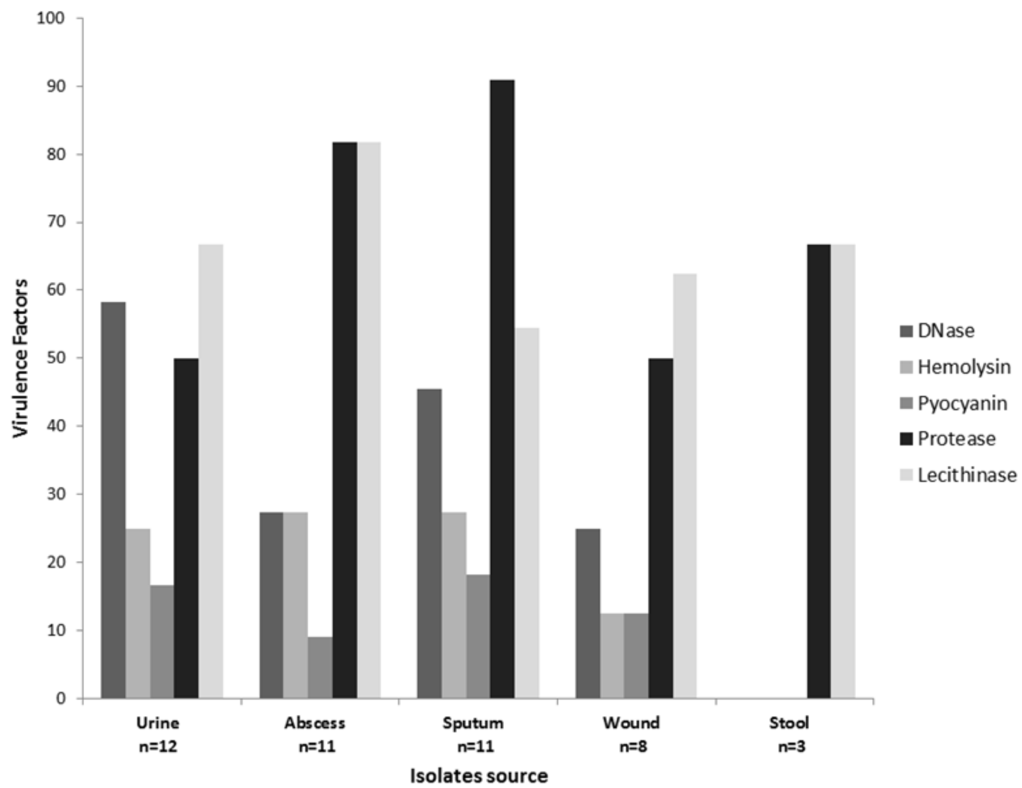

Figure 4. Distribution of different virulence factors in *P. aeruginosa* isolates according to the clinical sources.

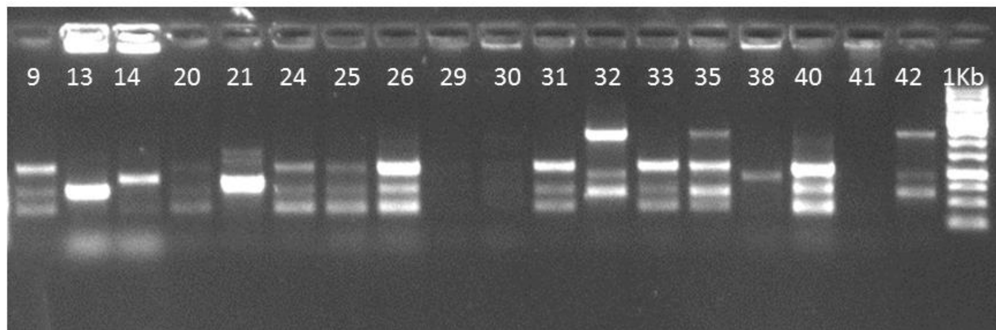

Figure 5. Representative DNA-banding profiles of *P. aeruginosa* isolates by agarose gel electrophoresis following RAPD PCR using primer 208.Lane 1-18: *P. aeruginosa* isolates, lane 19: 1 Kb DNA ladder.

Figure 6. Phylogenetic tree using primer 208.

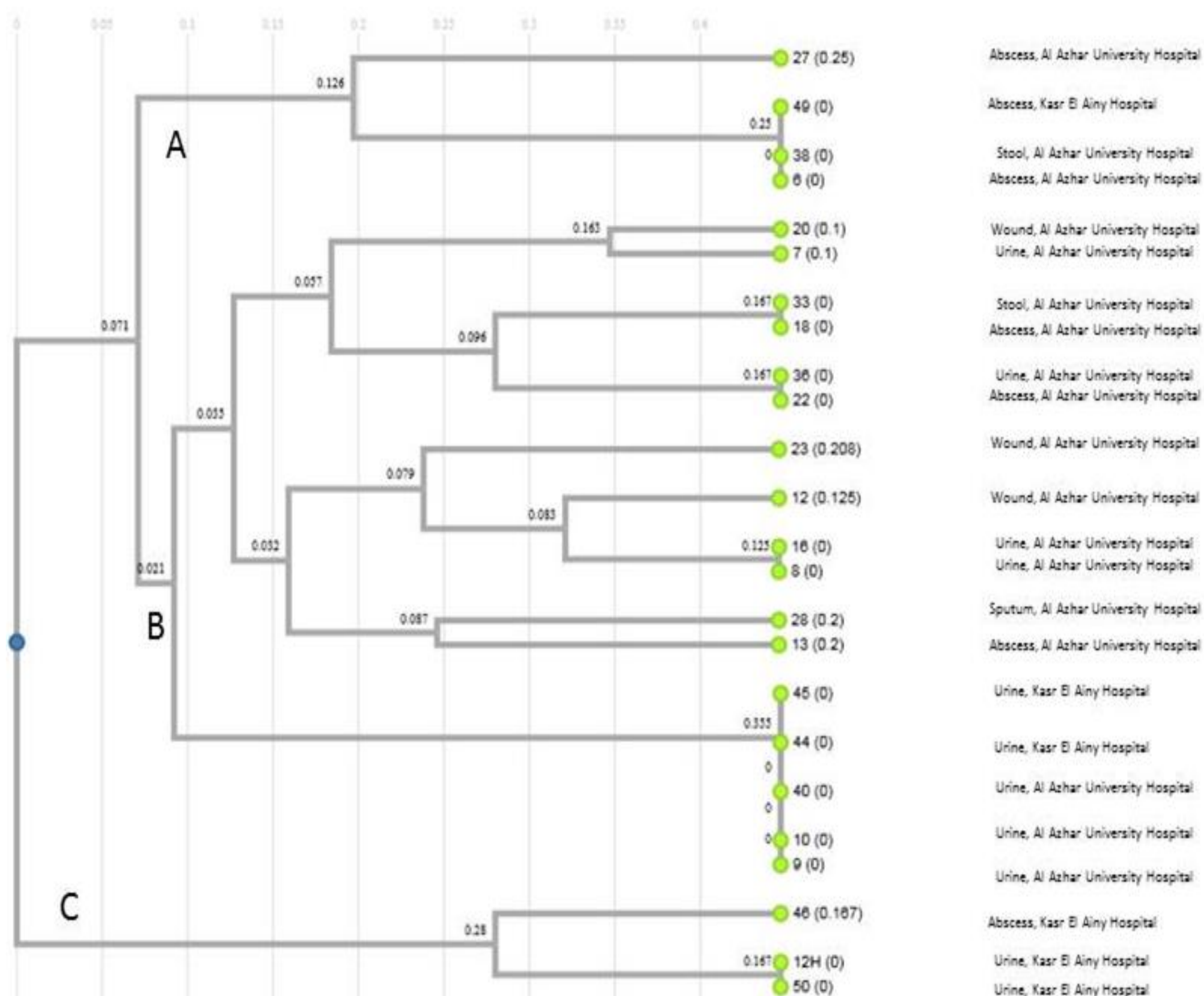

Figure 7. Phylogenetic tree using primer 1281.

sources. It also reveals that protease and lecithinase are the most common virulence factors produced by all isolates regardless of the isolation source. The common sources of infection in hospitals environment are obvious from the similar genotypic pattern of a large number of isolates. Further studies on a larger number of isolates are required to confirm our observations. We suggest applying strict disinfection policy in hospitals environment to prevent spreading of infections, in addition, new classes of antibiotics should replace traditional antibiotic treatment to prevent emergence of resistant strains of *P. aeruginosa*.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Akopyanz NN, Bukanov O, Westblom TU, Kresovich S, Berg, DE (1992).DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. 20:5137-5142.

Aly M, Balkhy HH (2012). The prevalence of antimicrobial resistance in clinical isolates from Gulf Corporation Council countries. Antimicrob. Resist. Infect. Control 1(1):26.

Al Mashhadani KA (2004). A study on diagnosis and pathogenesis of *P. aeruginosa* isolated from different sources in Mosul City. Ph.D. Thesis. Mosul University college of Science.www.iasj.net/iasj.

Bonfiglio G, Carciotto V, Russo G, Stefani S, Schito GC, Debbia E, Nicoletti G (1998).Antibiotic resistance in *P.aeruginosa*: an Italian survey. J. Antimicrob. Chemother. 41:307-310.

Campbell M, Mahenthiralingam, E, Speert DP (2000). Evaluation of random amplified polymorphic DNA typing of *P. aeruginosa*. J. Clin. Microbiol. 38(12):4614-4615.

Cevahir N, Demir M, Kaleli I, Gurbuz M, Tikvesli S (2008). Evaluation of biofilm production, gelatinase activity, and mannose-resistant hemagglutination *Acinetobacter baumannii* strains. J. Micro. biol.

Immunol. Infect. 41:513-518.
Ciofu O, Lee B, Johannesson M, Hermansen NO, Meyer P, Høiby N (2008). Scandinavian cystic fibrosis study consortium. Investigation of the algT operon sequence in mucoid and non-mucoid *P.aeruginosa* isolates from 115 Scandinavian patients with cystic fibrosis and in 88 *in vitro* non-mucoid revertants. Microbiology 154:103-113.
Clinical and Laboratory Standards Institute (CLSI) (2011). Performance standards for antimicrobial susceptibility testing.20th informational supplement.M100-S20.Wayne, PA, EE.UU.
Fatima A, Naqvi SB, Khaliq SA, Pervee S, Jabeen S (2012). Antimicrobial susceptibility pattern of clinical isolates of *P. aeruginosa* isolated from patients of lower respiratory tract infections. Springer Plus.1:70.
Fristch P, Hanson MA, Spore CD, Pack PE, Riseberg LH (1993).Constancy of RAPD primer amplification strength among distantly related taxa of flowering plants. Plant Mol. Boil. Res. 11:10-20.
Gales AC, Jones RN, Turnidge J, Rennie R, Ramphal R. (2001). Characterization of *P.aeruginosa* Isolates: Occurrence Rates, Antimicrobial Susceptibility Patterns, and Molecular Typing in the Global SENTRY Antimicrobial Surveillance Program, 1997–1999. J. Clin. Infect. Dis. 32:146-155
Graham J, McNicol RJ (1995).An Examination of the ability of RAPD Markers to Determine the Relationships within and between Rubus spp. Theor. Appl. Genet. 90:1128-1132.
Holban AM, Chifiriuc MC, Cotar AI, Bleotu C, Grumezescu AM, Banu O, Lazar V (2013). Virulence markers in *P.aeruginosa* isolates from hospital- acquired infections occurred in patients with underlying cardiovascular disease. Rom. Biotechnol. Lett. 18 (6):8843-8854.
Huerta V, Mihalik K, Crixell, SH, Vattem DA (2008).Herbs, Spices and Medicinal Plants used in Hispanic Traditional Medicine Can Decrease Quorum Sensing Dependent Virulence in *P. aeruginosa*. Int. J. appl. res. nat. prod. 1(2):9-15.
Hughes DT, Sperandio V (2008). Inter- kingdom signaling: communication between bacteria and their hosts. Nat. Rev. Microbiol. 6:111-120.
Jácome PR, Alves LR, Cabral AB, Lopes AC, Maciel MA (2012). Phenotypic and molecular characterization of antimicrobial resistance and virulence factors in *P.aeruginosa* clinical isolates from Recife, State of Pernambuco, Brazil. Rev. Soc. Bras. Med. Trop. 45(6):707-712.
Karp A, Edwards KJ (1997). DNA markers a global review. In: G. Caetano-Anolle's and P.M. Gresshoff (eds DNA Markers protocols, Application and overviews). Wiley, New York. pp. 1-13.
Kiewitz C, Tummler B (2000). Sequence diversity of *Psedomonas aeruginosa*: Impact on population structure and genome evolution. J. Bacteriol. 182:3125-3135.
Kumar NS, Gurusubramanian G (2011). Random amplified polymorphic DNA (RAPD) Markers and its applications. Sci. Vis. 11(3):116-124.
Lim KT, Yasin RM, Yeo CC, Puthucheary SD, Balan G, Maning N, Wahab, ZA, Ismail N, Tan EA, Mustaffa A, Thong KL (2009). Genetic fingerprinting and antimicrobial susceptibility profiles of *P.aeruginosa* hospital isolates in Malaysia. J. Micro. Biol. Immunol. Infect. 42(3):197-209.
Mahmoud AB, Zahran WA, Hindawi GR., Labib AZ, Galal R (2013). Prevalence of multidrug-resistant *P.aeruginosa* in patients with nosocomial infections at a University Hospital in Egypt, with Special Reference to Typing Methods. J. Virol. Microbiol. 2013.
Martin C, Boyd EF, Quetin P, Massicot, Selander RK (1999). Enzyme polymorphism in *P.aeruginosa* strains recovered from cystic fibrosis patients in France. Microbiology 145:2578-2595.
Matar GM, Chaar MH, Araj GF, Srour Z, Jamaleddine G, Hadi U (2005). Detection of a highly prevalent and potentially virulent strain of *P.aeruginosa* from nosocomial infections in a medical center. BMC Microbiol. 5:29.
Menon WG, Priya Eswaran SH, Pant SS, Bharadwaj R, Nagendra A (2003). Random amplification of polymorphic DNA based typing of *Pseudomonas aeruginosa*. Med. J. Armed Forces India. 59(1):25-28.
Nunes EL, Dos Santos KR, Mondino PJ, Bastos MD, Giambiagi-de Marval M (1999). Detection of *ileS*-2 gene encoding mupirocin resistance in methicillin-resistant *Staphylococcus aureus* by multiplex PCR. Diagn. Microbiol. Infect. Dis. 34:77-81.
Onasanya A, Basso A, Somado E, Gasor ER, Nwilene FE, Ingelbrecht I, Lamo J,Wydra K, Ekperigin MM, Langa M, Oyelakin O, Sere Y, Winter S, Onasanya RO (2010). Development a combined molecular diagnostic and DNA fingerprinting technique for rice bacteria pathogens in Africa. Biotechnology 9:89-105.
Pollak M (1998). Infections due to Pseudomonas species and related organisms in Adams, E, Braunwald, KJ, Isselbacher, JB, Martin, Wilson, JDpp: 156. McGraw-Hill.New jersey, USA.
Rumiy R, Genauzeau E, Barnabe C, Beaulieu A, Tibayrenc M, Andremont A (2001). Genetic diversity of *Psedomonas aeruginosa* strains isolated from ventilated patients with bacteremia and environmental water. Infect. Immun. 69(1):584-588.
Santo E, Macedo C, Marin, JM (2006). Virulence factors of uropathogenic *Escherichia coli* from a university hospital in Ribeirão Preto, São Paulo, Brazil. Rev. Inst. Med. Trop. São Paulo. 48: 185-188.
Schaechter M, Baldauf SL, Baross JA, Baulcombe DC, Haselkorn R, HopWood DA, Ingraham JL (2009). Encyclopedia of Microbiology.pp: 314. 3 ed. AP Press. USA.
Simonetti A, Ottaaiano E, Diana MV, Onza C, Triassi M (2013). Epidemiology of hospital acquired infections in an adult intensive care unit: results of a prospective cohort study. Ann. Ig. 25(4):281-289.
Todar K (2009). The mechanisms of bacterial pathogenicity. Todar's Online Textbook of Bacteriology. [Internet].Available from: http://textbookofbacteriology.net/pathogenesis.html
Van Delden C, Iglewski BH (1998). Cell to-Cell signaling and *P.aeruginosa* infections. Emerg. Infect. Dis. 4(4):551-560.
Williams JG, Kubelik AR, Livak, KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res.18:6531-6535.
Yayan J, Ghebremedhin B, Rasche K (2015).Antibiotic Resistance of *P.aeruginosa* in Pneumonia at a Single University Hospital Center in Germany over a 10-Year Period. PLoS One. 10(10):e0139836.
Zenone T, Souillet G (1996). X linked Agammaglobulinemia presenting as *P.aeruginosa* septicemia. Scand. J. Infect. Dis. 28:417-418.
Zulkifli Y, Alitheen NB, Son R, Raha AR, Samuel L, Yeap SK, Nishibuchi M (2009). Random amplified polymorphic DNA-PCR and ERIC PCR analysis on *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. Int. Food Res. J.16: 141-150.

academicJournals

Vol. 10(39), pp. 1654-1661, 21 October, 2016
DOI: 10.5897/AJMR2016.8273
Article Number: 490D61061249
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Bacterial association and oral biofilm formation: A bibliometric analysis

Luana Kelle Batista Moura[1]*, Fabrício Ibiapina Tapety[1,2], Mitra Mobim[1], Eliana Campelo Lago[1,3], Eduardo Souza de Lobão Veras[1], Carla Maria de Carvalho Leite Leal Nunes[1,4], Tanit Clementino Santos[1] and Thiago Lima Monte[1]

[1]Post Graduation Program, Professional Masters in the Family Health, University Center UNINOVAFAPI, Teresina, Piauí, Brasil.
[2]State University, Piauí - UESPI, Teresina, Piauí, Brasil.
[3]State University, Maranhão - UEMA, Teresina, Piauí, Brasil.
[4]Federal University, Piauí - UFPI, Teresina, Piauí, Brasil.

Received 18 August, 2016, Accepted 11 October, 2016.

Biofilms are complex aggregations of microorganisms formed in any situation where there is contact with superfaces solids, liquids or gases. This study aims to map the international scientific literature on the formation, bacterial adhesion, prevention and treatment of oral biofilms. There were 921 published articles in 274 different journals indexed in the Web of Science, in the 1991-2015 year period. These articles were written by 2804 authors linked to 695 institutions in 59 countries, notably the United States and the United Kingdom. The 17 articles of greater impact were published in the 1995-2009 year period. The relationship between the most important articles shows that the issue is addressed in a broad and diversified approach, points out to a range of possibilities for prevention and treatment, and uses of experimental methods that list mechanisms and combinations able to enter the structure of the biofilm, elucidate the formation process, to bacterial adhesion and to reduce pathogenic bacterial activity and, in some cases, destroying their structure. The descriptive analysis of the main work has shown potential for the development of the area and contributions to improve the prevention and treatment of oral biofilms.

Key words: Dental plaque, biofilm, bibliometric analysis.

INTRODUCTION

Biofilms are complex aggregations of microorganisms and are formed in situations where there is contact of solids superfaces with liquids or gases. In infectious processes, the bacterials may be present in two ways: as free-living or sessile cells. This second stage of life of the bacteria is seen in microscopic and biological systems with high level of organization called biofilms. These micro-ecosystem, may comprise one or more species of

*Corresponding author. E-mail: luana_moura19@hotmail.com. Tel: 55 - 086 - 99982-8280.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

bacteria producing films that are deposited and fixate on biotic and abiotic surfaces, forming organized and functional aggregates, allowing a strong adhesion and constitute a defensive mechanism facing hostile environments (Elias and Banin, 2012; Li and Tian, 2012).

The biofilm allows adhesion of bacterial cells to an abiotic surface very quickly, and has been found that an affinity between the organic material and polymeric and hydrophobic surfaces. In the biotic surfaces, adhesion occurs more slowly, mediated by specific processes. The organic matrix, organized in layers produced by these bacteria and gives them greater resistance to anti-microbials, make it difficult to eradicate the source of infection. Several features have been identified as responsible for tolerance to antimicrobials: the protection of proteins and compression of the matrix, low metabolism, resistance genes transfer between species and failure to recognize these structures by the immune system. Another factor considered of impact in this phenomenon is the indiscriminate use of antibiotics, which induce bacterial resistance, increasing the concentrations necessary for combating these micro-organisms and widening the search for new drugs (Preedy et al., 2014; Krasowska and Sigler, 2014; Patel et al., 2015)

The biofilm formation does not occur randomly. The construction of this structure and dispersion of this system are regulated by quorum-sensing, communication between cells dependent on population density. Microorganisms disperse signaling molecules and detect the amount of other microorganisms near them. They are also sensitive to external factors and alter the signaling based on them. Then, there is a communication between species allowing formation of associations and more resistant biofilms (Popat et al., 2012).

Dental plaque or oral biofilm, is a complex biofilm that may be accumulated on the tooth surface or gum and is directly related to periodontal disease and tooth decay (Simoes et al., 2009). For the other hand, the the bibliometric research is a quantitative and statistical technique that concentrates its efforts on visible indicators and academic activity goals, usually of publications and citations (Cronin, 2001). The relevance of bibliometric analysis as a technique for data collection and analysis have been confirmed as one of the argumentative sources in the search for investment resources in research, in academic rankings (Diem and Wolter, 2013; Miguel and Dimitri, 2013). Considering the relevance of bacterial association and formation of oral biofilms and their role in cariogenic and periodontal diseases, this study aimed to map the international scientific literature on the formation, bacterial adhesion, prevention and treatment of oral biofilms.

METHODOLOGY

To carry out a bibliometric study, important selection of the database, keeping compatibility of this choice with the research objectives and the achievement of results was done (Koskinen et al., 2008).

It was specified as database ISI Web of Knowledge/Web of Science for its academic recognition to be considered one of the most comprehensive regular basis covering various areas of scientific knowledge (Santos et al., 2011). The steps for the analysis of selected studies followed the three suggested procedures: the definition of the database and the criteria to be used for the collection; data collection; and the representation and data analysis (Santos et al., 2014).

To collect data, the database of the Web Science™ was used in time range of 1945 to 2015, to allow replication or update this study without the need to carry it again from its beginning. The following search terms were defined: "dental plaque*" and "biofilm*". The quotation marks indicate the exact representation of terms with more than one word and asterisks, the descriptors plural possibilities. These three terms represent the intended association in compliance with the purpose of the study. The data was collected from the search of these terms in the "topic" which is the title of articles, abstracts, keywords, author and keywords created (keywords plus). The results showed the first publication record in 1991.

After the search, there were identified 921 records publications, which were used as set searches for bibliometric analysis proposed in this study. There was no refinement filter to areas of knowledge, countries or languages of the studies, including all records of publications that had the three terms in association.

Later, data collection was performed for the analysis of the material from the export of this data to the bibliometric analysis software package HistCite™ in order to organize information and facilitate analysis. The trajectory of annual evolution of publications; Periodicals with higher records; the authors with the highest number of publications; the number of articles distributed by country of origin of the authors; the more articles cited in Web of Science (global) and those most cited in the set of selected items (local), were analyzed. In addition, to these data generated by the software were identified general aspects of the texts of the 17 articles that compose the two groups: (a) items that received more citations from other papers throughout the database ISI Web of Science™ (GCS) and (B) articles that received more citations of the works in the selection group this bibliometric study (LCS).

RESULTS

921 publications records on formation, bacterial adhesion, prevention and treatment of oral biofilm in the main collection of Web Science™ were identified. These articles were published in 274 different journals indexed to the database and were written by 2804 authors with associations to 695 institutions in 59 countries. To achieve these items, 22,238 references, with an average of approximately 24 references per article were used as demonstrated in Table 1.

The first publication record, dating from 1991 shows experiments that studied factors that influence the capacity for microbial colonization and interactions in biofilms under controlled conditions (Donoghue and Perrons, 1991). The apex of the number of publications was reached in 2015, indicating a growing trend in the interest in this subject, in addition to timeliness and relevance of the subject. The growth percentage of publications in the period 1991-2015 was 8.900%. The

Table 1. Bibliometric analysis-Biofilm / Dental Plaque - publications (1991-2015).

Bibliometric data	Amount
Publications (articles)	921
Indexed journals	274
Authors	2804
Institutions (associations of the authors)	695
Countries	59
Cited References	22.238

Source: Elaborated from the Web of Science data.

Figure 1. Distribution of publications on dental plaque/biofilm (1991-2015). Source: Elaborated from the Web of Science data.

evolution of the number of publications distributed by years of the records can be seen in Figure 1.

The total quantitative of publications was organized by the corresponding periodic conform in Table 2. The journals with the largest number of published records are the "Archives of oral biology" and "Caries Research", both with 43 articles. However, to identify those journals with the highest impact, was defined an index by dividing the number of citations by the number of published papers, presenting the "Journal of Bacteriology" as the one with the highest rate (79.6). This information become relevant for researchers and research centers to map the academic journals that publish most in the thematic and receive more citations from other studies.

The authors with the highest number of publications were presented in Table 3. Among the 2804 authors identified in the study, Table 3 shows the ten with the highest number of publications. The first is Michael Wilson, a researcher at the post-graduate school in dentistry in Europe, where he also works with Jonathan Pratten. The observation of Table 3 allows the identification of at least three major research centers in the area, "UCL Eastman Dental Institute – United Kingdom," the "University of Otago Wellington – New Zealand" and the "University of Maryland – United States of America", all research centers with a minimum of two researchers and more than forty records of publications. Furthermore, the specific situation in the area of knowledge of dental studies localizes the results in a perspective that corroborates with the initial interest in this study. The quantity of articles by country of origin of the membership of the authors is presented in Table 4.

Table 2. Top journals with more published articles on dental plaque/biofilm (1991-2015).

Journals	Quantity of articles	Citations	Citations/ Quantity
Archives of oral biology	43	1028	23,90
Caries research	43	1289	29,97
Journal of dental research	40	1264	31,6
Oral microbiology and immunology7	35	893	25,51
Applied and environmental microbiology	33	1258	38,12
Journal of clinical periodontology	26	1345	51,73
Journal of dentistry	21	286	13,61
BMC microbiology	20	339	16,95
FEMS microbiology letters	20	753	37,65

Source: Elaborated from the Web of Science data.

Table 3. Authors with more publications on dental plaque/biofilm (1991-2015).

Authors	Quantity of articles	Membership (Institution with Affiliation)	Country
Wilson M	31	UCL Eastman Dental Institute	United Kingdom
Sissons CH	26	University of Otago Wellington	New Zealand
Marsh PD	24	University of Leeds	United Kingdom
Kolenbrander PE	21	University of Mariland	United States of America
Pratten J	21	UCL Eastman Dental Institute	United Kingdom
Xu HHK	20	University of Mariland	United States of America
Wong L	19	University of Otago Wellington	New Zealand
Scannapieco FA	18	University at Buffalo School of Dental Medicine	United States of America
Weir MD	17	University of Mariland	United States of America
Lamont RJ	16	University of Louisville	United States of America

Source: Elaborated from the Web of Science data.

Table 4. Number of articles by origin country and author membership.

Country	Quantity
United States of America	316
United Kingdom	155
Brazil	78
China	73
Japan	71
Netherlands	56
Germany	50
New Zealand	35
Sweden	32

Source: Elaborated from the Web of Science data.

Table 4 indicates the countries of work memberships, with predominance in studies of American origin, followed by the UK and Brazil. It is important to note that the results coincide with the list of authors with most publications only to the first two countries, as Brazil is not in the list shown in Table 3. This may be due to the fact that Brazilian works are more decentralized in authoring or not have specialized centers on the field, despite the amount of individual records.

To analysis the relationship between the most cited articles, the records were separated into two groups: (a) items that received more citations from other papers throughout the database ISI Web of Science^{TM} (GCS) and (b) articles that received more citations of the works in the selection group in this bibliometric study (LCS). The number of citations and citation relations between these works (represented by lines connecting the circles) indicate the most representative studies on the subject, presenting seminal works and those that later were also very cited (Figure 2).

By observing the relationship between the texts, entitled figures can be identified "authority article "or "base article" (Santos et al., 2014), which are those main references of others who receive large amounts of citations. Are they: Kolenbrander (2000), Loo et al. (2000)

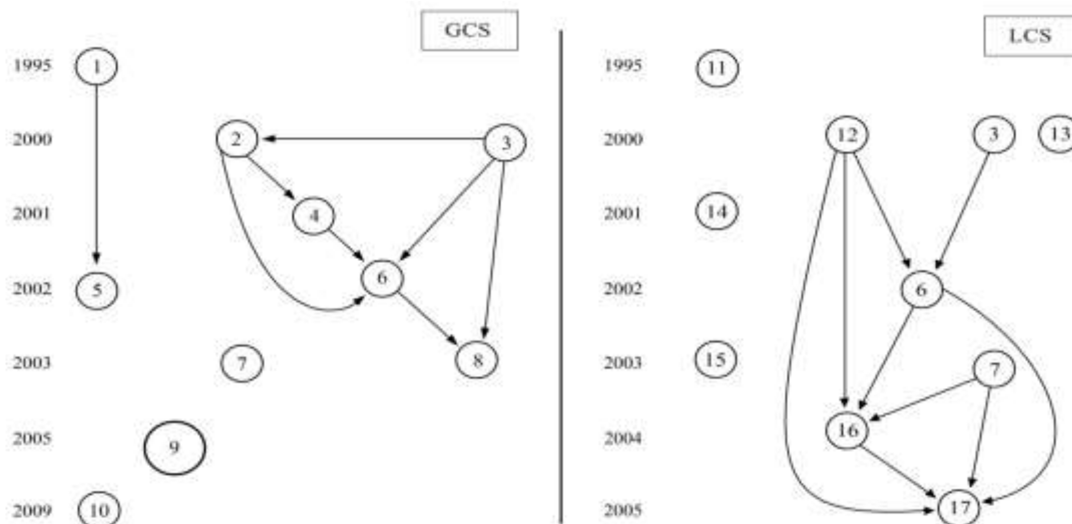

Figure 2. Top 10 most cited articles in Web of Science (Global Citation Score) and most cited by the selected items (Local Citation Score), from the field selection (1991-2015). Source: Elaborated from the Web of Science data. 1– Quirynen and Bollen (1995); 2– Kolenbrander (2000); 3– Loo et al., (2000); 4– Li et al., (2001); 5– Quirynen et al. (2002); 6– Kolenbranderet al (2002); 7– Marsh (2003); 8– Rickard et al. (2003); 9– Pihlstrom et al. (2005); 10 – Hall-Stoodley and Stoodley (2009); 11 – Marsh and Bradshaw (1995); 12 – Wood et al,2000); 13– Rosan and Lamont (2000); 14– Guggenheim et al. (2001); 15– Palmer et al. (2003); 16– Marsh (2004); 17– Marsh (2005).

and Wood et al. (2000). In addition, the "hub articles" or "Connecion articles", which are those that condense important information from previous work by connecting these to other more recent, also receiving large amounts of citations (Santos et al., 2014), They are: Li et al. (2001), Kolenbrander et al. (2002) and Marsh (2004). Older articles of the relationship shown in Figure 1 are: Marsh and Bradshaw (1995) and Quirynen and Bollen (1995).

DISCUSSION

The study is aimed at evaluating the recent advances in microbiology of dental plaque, with particular emphasis on the mechanisms by which the bacteria adhere to the tooth surface and produce a biofilm. It also describe the formation of biofilm on the tooth surface and cites caries and periodontal diseases that arise due to the formation of dental plaque. The study shows that bacteria associated with biofilm formation are more resistant to antibiotics as compared to suspended molecules and suggests that it is necessary to intervene in the formation and development of biofilms. The principal contributions lie in the presentation of the use of some strategies in an attempt to reduce the bacterial activity in the biofilm, such as the use of fluoride, fissure sealant that reduces acid production, combined use of anti-plaque and anti-bacterial that only inhibits the formation of some microorganisms, and vaccination which can raise antibodies against a specific bacterial type (Marsh and Bradshaw, 1995).

The second study aimed to examine the influence of roughness and surface free energy in the bacterial adhesion process. The authors sought scientific evidence on bacterial adhesion to oral surfaces. As a result, it was Quirynen and Bollen, (1995) that evidenced that the roughness of oral surfaces has a direct impact on bacterial adhesion and biofilm formation and maturation. The increase in surface roughness and free energy directly influenced the bacterial adhesion, plaque formation, thus contributing to the occurrence of future charitable processes and periodontal infections (Quirynen and Bollen, 1995).

Authority article number two, in the shown relationship in Figure 1 (GCS), explores the complexity of the process of biofilm formation and maintenance, as regards the genetic composition, types and multiple species of bacteria groups. This study also emphasizes how future perspectives, the association of new technologies such as electronic confocal laser microscopy to investigate the communication between bacteria and demonstrate more objectively this phenomenon, the formation of biofilms (Kolenbrander, 2000).

One of the authority articles, number three, represented in Figure 1, (GCS), characterized the biofilm formation of *Streptococcus gordonii* in vitro to identify formation of biofilms with genetic defects. In the experiment, the formation of biofilms was altered by changes in pH, osmolality and carbohydrate content. The results suggest that bacterial physiology has a fundamental role in biofilm

formation. The researchers believe that the study of genes can provide information that will assist the understanding of biofilm formation process of oral streptococci (Loo et al., 2000).

The contribution of this study sums up the importance of isolating defective biofilms (mutants) for the understanding of mechanisms for control and prevention of biofilm from the identified defects in the genes. The authors support the idea that understanding the processes involved in the biofilm formation and development through studies of the genes associated with them can provide strategies for the control and prevention of infections mediated by biofilms. This may well trigger the development of agents to combat effectively the bacterial association (Loo et al., 2000).

Study represented in Figure 1 (GCS), with the number four, investigated the biofilm formation by *Streptococcus mutans* and considered the results through genetic analysis. It showed that the *S. mutans* grown in biofilm is more successful. Furthermore, dead cells in the biofilm can serve as donor material resistant to antibiotics. *S. mutans* have higher development capability within the biofilm and is even able to absorb genetic material of dead individuals of the same species, including transferring material resistant to antibiotics which conferring greater resistance to the biofilm (Li et al., 2001). A literature review, represented in Figure 1 (GCS), with the number five, sought risk of infection in cases of oral implant. One of the mentioned risks is the accumulation of bacteria by plaque formation. Analyzed studies indicate that, in studies performed both in animals and in humans, the microflora in the case of implants is similar to that present in periodontitis. However, the authors pointed out that the formation of plaque is not the only risk for infection, but the presence of smoking and poor oral hygiene contributes greatly to the development of infection in oral implants. In the case of implants, it is necessary that the patient undergoes a screening prior to minimize the risk of acquiring infection (Quirynen et al., 2000). This work (Quirynen and Bollen, 1995) mentioned that *in vivo* studies have examined the surface roughness on plaque formation and the relationship with periodontal inflammation.

Article authority, number six, the relationship shown in Figure 1 (GCS) on the communication between the oral bacteria, citing Article Two (Kolenbrander, 2000), shows that oral bacteria interact with their environment, form mixed-species communities and adhere to the surface. Many of the physical interactions that occur between organisms of this community are known. The adhesins are of various molecular sizes and species that support adhesins compete with each other for binding to the polysaccharide receptor. Thus, it is postulated that the co-aggregation and co-adhesion are essential for communication between species and help establish spatio-temporal patterns of development (Kolenbrander et al., 2002) Citing the articles three (Loo et al., 2000) and four (Li et al., 2001), shows that the possibility of oral streptococci in biofilms has been verified and can communicate via a signaling and detection system. A signaling system is essential for genetic competence in *S. mutans* and is involved in biofilm formation (Kolenbrander et al., 2002).

Study discusses the properties of dental plaque in order to elucidate its composition and the relationship between the biofilm and the oral microflora. The author considers the hypothesis that biofilms can be combated not only directly with the impediment of its formation, for example, but also through environmental relations between microorganisms. Contribution to the knowledge on the prevention and treatment of the biofilm is the possibility of action for understanding and working knowledge of oral microflora, this why treatments are restricted to combat the symptoms without worrying about the ecological characteristics of the mouth. With the understanding of the peculiarities of oral flora, a holistic care becomes feasible (Marsh, 2003).

In the research on the processes of coaggregation, it was found that genetically distinct bacteria join together by means of specific molecules to form complex aggregates and give origin to complex biofilms. The researchers suggest that there is evidence that these formations are possible in dental plaque in which occurs such junction between different bacteria and biofilm formation. The coaggregation among different species gives greater resistance to the biofilms (Rickard et al., 2003).

Then, the relationship presented in Figure 1, in Article nine (Pihlstrom et al., 2005), discuss the factors that cause periodontal diseases, especially gingivitis caused by biofilms which are related to other factors such as the use of tobacco which contribute to the development of gingivitis. The authors state that, to treat periodontal disease, it is necessary to control the biofilm and other risk factors. Further, the diseases may be treated with systemic antibiotics. But with the use of these, there is a risk of provoking the development of resistant microorganisms. It also point out that the greatest risk factor for periodontal diseases are the biofilms that form when oral hygiene is poor. In this work, the most common ways to prevent and treat biofilms are: oral hygiene, flossing and mouthwash.

The last study presented at the global graph of citations, number ten, investigates attempts to analyze the characteristics of biofilms (phenotype) of different bacterial species. The authors analyze several studies through a review and suggest that there are few investigations that seek to understand how the biofilm interferes with infectious diseases in humans. Understanding the inflammatory response of the host is now been understood. The authors believe that clinical studies of infections caused by biofilms are relevant to understanding of the interaction between bacteria and biofilm in the host's inflammatory response. From new

studies, it will be possible to review the strategies of prevention and treatment of biofilm (Hall Stoodley and Stoodley, 2009).

The first authority article in the relationship shown in Figure 1 (LCS), the local citation graph contains as research problem, the removal of intact plate so that the biofilm can be viewed entirely in the laboratory, or in its natural state and integral to the identification of possible structures or spaces that could be useful in understanding and combating biofilm. The authors conclude that knowing the biofilm structure with their respective spaces is fundamental to the application of therapies to desired targets within the plaque. Circulatory channels have been identified in dental plaque that can function as a path for application of therapeutic agents within the biofilm. The authors also suggest that there are common features in all bacterial biofilm structures, thus this study is very important because it can provide the developing of new biofilm treatments (Wood et al., 2000). Still in 2000, another study, represented by the number thirteen in Figure 1 (LCS), is a review which discusses the molecular basis of bacterial adhesion to tooth surface. The adhesion process provides stability of bacterial structures and is performed by a multitude of molecules that have critical roles within the biofilm structure. The adhesin, as a adherence protein complex, will define the success or failure of the dental plaque adhesion, to rely on their performance at the time of bacterial adhesion (Rosan and Lamont, 2000).

In the following year, a study represented by the number fourteen in Figure 1 (LCS), describes a supragingival plaque model grown in the laboratory containing the bacteria: *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum*, *Streptococcus sobrinus* and *Streptococcus oralis*. In the experiment, researchers developed a biofilm model very useful for preclinical testing of agents to combat biofilms. It was introduced in the experiment chlorhexidine and was observed that there were significant losses on the viability of the biofilm similar to losses *in vivo*. It was still possible to observe the antibacterial effects of chlorhexidine. The search is important for bacterial adhesion studies and coaggregation (Guggenheim et al., 2001).

Isolated in the figure, but very cited among the group object of research articles, the article number fifteen Figure 1 (LCS) tests the hypothesis that the adhesion between bacteria influence the development of plaque. The study is the first to demonstrate interactions mediated by coaggregation of bacteria during the initial phase of the plaque. There is a relationship between communities of mixed species and early plaque formation. The study is an example to further investigate, isolate and characterize bacterial interactions during the initial stage of plaque (Palmer et al., 2003).

By means of advanced technology, it was identified in article represented by the number sixteen in Figure 1 (LCS), that the biofilm has void spaces, and channels. The study focuses on understanding the plaque as a biofilm of mixed cultures in order to propose new strategies for controlling biofilms. Such understanding will result in future studies on more effective agents for inhibiting plaque, mechanisms able to interfere with the communication and bacterial degradation of the biofilm structure and identification of pathogenic clones may improve diagnosis and predict disease susceptibility (Marsh, 2004).

Study represented by the number seventeen in Figure 1 (LCS), shows that biofilms are complex structures organized and populated by communities of micro-organisms that communicate through gene transfer or through molecules. Through a review, the author evaluates whether the plaque exhibits properties consistent with those of a typical microbial biofilm communities. The results show that the plaque has a structure with an extracellular matrix of a different composition and communication are performed by means of gene transfer, such organization increases the success of the plaque as it increases it's metabolic efficiency, gives greater strength and increased virulence, that is, the plaque exhibits characteristics that are typical of biofilms and microbial communities in general (Marsh, 2005).

Based on other articles (Marsh, 2003, 2004), dental plaque is defined as a multiple community of microorganisms as a biofilm, this community is embodied by an extracellular matrix of bacterial polymers (Marsh, 2005).

The twelfth study of Figure 1 (LCS) demonstrates that microscopic techniques have been developed to investigate biofilms and show that the plaque behaves like a typical biofilm (Wood et al., 2000). The article represented by the number seventeen of Figure 1 (LCS) corroborates to the results that indicate that the plaque has a structure containing pores or channels that point areas where there is interaction at the interface tooth (Marsh, 2005). When citing the work represented by the number six of Figure 1 (LCS), how the communication between the organisms of the plate was carried out by means of small molecules, was explained.

It is noticed that, as shown in Figure 1, - Citation Score - GCS, the top 10 most cited article in the Web of Science, was number nine of the authors, Pilhlstrom et al. (2005), which sought evidence of bacterial coaggregation and development of multi-species biofilm, with 976 citations throughout the database. Top 10 most cited article in The Local Citation Score - LCS, was the work of Kolenbrander et al. (2002), which sought evidence of communication among the oral bacteria, with 77 citations within the selection carried out in the set of articles published in the period 1991 to 2015 on training, bacterial adhesion, prevention and treatment of oral biofilms. Interestingly, the temporal gap, even in a cut that includes recent articles, those most cited have over 10 years of publication and this may be indicative of a

maturation period of the results and the interstices between its release and subsequent citations it receives.
The international literature dealing with the subject in a broadly diversified way, points to a range of possibilities for the prevention and treatment of oral biofilms, as well as experimental methods and possibilities of use in studies for this purpose. The prevention of oral biofilm is a complex process because it involves several species of microorganisms in their composition. The studies analyzed the importance of understanding how formation occurred and observed bacterial adhesion. From this understanding, it is possible to list effective strategies to prevent the development of oral biofilms.

Conclusion

There were 921 published articles in 274 different journals indexed in the Web of Science, in the 1991-2015 year period, on training, bacterial adhesion, prevention and treatment of oral biofilms. These articles were written by 2804 authors with ties to 695 institutions in 59 countries, notably the United States and the United Kingdom. The 17 articles of greater impact were published in the 1995 to 2009 year period.

The substances that penetrate and destroy the structure of the biofilm have been already discussed in articles by means of *in vitro* experimental studies that have been listed for mechanisms and combinations able to penetrate the biofilm structure and reduce pathogenic bacterial activity and, in some cases, destroy bacterial structure.

The scientific evidence on this subject can contribute to the development of new research in order to fill gaps in knowledge on the prevention and treatment of oral biofilm and implement effective public policies of oral health care.

Conflict of Interests

The authors have not declared any conflict of interestS.

REFERENCES

Cronin B (2001). Bibliometrics and beyond: some thoughts on web-based citation analysis. J. Inf. Sci. 27(1):1-7.

Diem A, Wolter SC (2013). The use of bibliometrics to measure research performance in education sciences. J. High. Educ. 54(86):86-114.

Donoghue HD, Perrons CJ (1991). Effect of nutrients on defined bacterial plaques and Streptococcus mutans C67-1 implantation in a model mouth. Caries Res. 25(2):108-115.

Elias S, Banin E (2012). Multi-species biofilms: living with friendly neighbors. FEMS Microbiol Ecol. 36(5):990-1004.

Guggenheim B, Giertsen ESCH, Schüpbach P, Shapiro S (2001). Validation of an *in vitro* biofilm model of supragingival plaque. J. Dent. Res. 80(1):363-370.

Hall SL, Stoodley P (2009). Evolving concepts in biofilm infections. Cell Res. 11(7):1034-1043.

Kolenbrander PE (2000). Oral microbial communities: Biofilms, interactions and genetic systems 1. Annu Rev Microbiol. 54(1):413-437.

Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ (2002). Communication among oral bacteria. Microbiol. Mol. Biol. Rev. 66(3):486-505.

Koskinen J, Isohanni M, Paajala H, Jääskeläinen E, Nieminen P, Koponen H, Miettunen J (2008). How to use bibliometric methods in evaluation of scientific research? An example from Finnish schizophrenia research. Nord. J. Psychiatry. 62(2):136-143.

Krasowska A, Sigler K (2014). How microorganisms use hydrophobicity and what does this mean for human needs? Front. Cell. Infect. Microbiol. 4(112):1-7.

Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001). Natural genetic transformation ofstreptococcus mutans growing in biofilms. J. Bacteriol. 183(3):897-908.

Li YH, Tian X (2012) Quorum sensing and bacterial social interactions in biofilms. Sensors. 12(3):2519-38.

Loo CY, Corliss DA, Ganeshkumar N (2000). Streptococcus gordonii biofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. 182 (5):1374-1382.

Marsh PD, Bradshaw DJ (1995). Dental plaque as a biofilm. J. Ind. Microbiol. 15(3):169-175.

Marsh PD (2003). Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub-and supragingival environment. *Oral* Dis. 9 (s1):16-22.

Marsh PD (2004). Dental plaque as a microbial biofilm. Caries Res. 38(3):204-211.

Marsh PD (2005). Dental plaque: biological significance of a biofilm and community life-style. J. Clin. Periodontol. 32 (s6): 7-15.

Miguel S, Dimitri, P (2013). La investigación en bibliometría en la Argentina: quiénes son y qué producen los autores argentinos que realizan estudios bibliométricos. Inf. Cult. Soc. (29):117-138.

Palmer-Júnior RJ, Gordon SM, Cisar JO, Kolenbrander PE (2003). Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. J. Bacteriol. 185 (11):3400-3409.

Patel VK, Gupta A, Singh D, Kant R, Bhattacharya S (2015). Surface Functionalization to Mitigate Fouling of Biodevices: A Critical Review. Rev Adhes Adhesives. 3(4):444-478.

Pihlstrom BL, Michalowicz BS, Johnson NW (2005). Periodontal diseases. Lancet. 366 (9499):1809-1820.

Popat R, Crusz SA, Messina M, Williams P, West SA, Diggle SP (2012). Quorum-sensing and cheating in bacterial biofilms. Proc Biol Sci. 279(1748):4765-4771.

Preedy E, Perni S, Nipiĉ D, Bohinc K, Prokopovich P (2014). Surface roughness mediated adhesion forces between borosilicate glass and gram-positive bacteria. Langmuir. 30(31):9466-76.

Quirynen M, Bollen CML (1995). The influence of surface roughness and surface-free energy on supra-and subgingival plaque formation in man. J. Clin. Periodontol. 22(1):1-14.

Quirynen M, De Soete M, Van Steenberghe D (2002). Infectious risks for oral implants: a review of the literature. Clin. Oral Implants Res. 13(1):1-19.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. Curr. Trends Microbiol. 11(2):94-100.

Rosan B, Lamont RJ (2000). Dental plaque formation. Microbes Infect. 2(13):1599-1607.

Santos JLS, Uriona Maldonado M, Santos, RNM (2011). Mapping of Academic and Scientific Publications on Organizational Memory. In: National Meeting of the National Association of Graduate Studies and Research in Administration, Rio de Janeiro, Brazil, P 43.

Santos JLS, Kalsing M, Hansen PB (2014). Interorganizational cooperation networks: a systematic review of the scientific production in the Web of Science 1981-2013. In: Proceedings of the XVII Seminars Administration; São Paulo, Brazil: FEA-USP; 2014. pp. 1-16.

Simoes M, Simoes LC, Vieira MJ (2009). Species association increases biofilm resistance to chemical and mechanical treatments. Water Res. 43(1):229-237.

Wood SR, Kirkham J, Marsh PD, Shore RC, Nattress B, Robinson C (2000). Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. J. Dent. Res. 79(1):21-27.

academicJournals

Vol. 10(39), pp. 1662-1667, 21 October, 2016
DOI: 10.5897/AJMR2016.8238
Article Number: 357C0D961251
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Campylobacter in sheep, calves and broiler chickens in the central region of Algeria: Phenotypic and antimicrobial resistance profiles

Meryem Guessoum[1]*, Zehor Guechi[2], Fares Aigoun[3], Sadjia Mahrane[2] and Amina Hachemi[1]

[1]National Higher Veterinary School, BP 161 Hacène Badi, El Harrach, Algiers, Algeria.
[2]Central Laboratory of Clinical Biology, University Hospital Center of Nafisa Hamoud (Parnet), Hussein Dey, Algiers, Algeria.
[3]Veterinarian Private, Bouira, Algeria.

Received 28 July, 2016; Accepted 6 September, 2016

The study was conducted in four slaughterhouses in Bouira Province, central region of Algeria. *Campylobacter* in the main food animals (sheep, calves and broilers) were studied to evaluate the prevalence, phenotypic characteristics and antibiotic susceptibility of isolated strains. Out of 200 sheep, 200 calves and 100 broilers swab samples collected. 150 strains were isolated and identified. A study of sensitivity to 14 antibiotics by the disc diffusion method was performed. This finding shows that, *Campylobacter* species are very common in avian samples isolates of (96%) but less frequent in sheep and calves (13 and 14% respectively). On the entire isolated strains, *Campylobacter jejuni* was the most common with an isolation rate of 58% followed by *C. coli* and *C. lari*. The majority of isolated *Campylobacter* strains showed as multidrug resistant. High rates of resistance to different antibiotics tested were observed in broilers, mainly to Nalidixic acid (96.8%), Ciprofloxacin (91.6%) and Erythromycin (88.54%); the lowest level of resistance was found to the Tetracycline (44.7%). The high frequency of digestive portage noted in food animals and the high rate of antibiotic resistance constitutes a real threat to public health in study area. In conclusion, significant *Campylobacter* isolation rate and multiple drug resistance should be at acceptable level so as to increase productivity livestock rearing off the study sites.

Key words: *Campylobacter*, sheep, calves, broiler, frequency, antibiotic resistance, Algeria.

INTRODUCTION

Campylobacter germs are a leading cause of enteric zoonotic infections (OIE, 2005; WHO, 2012). Poultry is generally considered to be the most important single reservoir for *Campylobacter*, mainly *Campylobacter jejuni* (Hakkinen et al., 2007). Contamination of chicken carcasses by *this germ* often occurs during the

*Corresponding author. E-mail: myriam.guessoum@gmail.com Tel: (+213) 0550554000.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

slaughtering process and consumption of chicken meat is a significant source of human *Campylobacter* infections (Lupo et al., 2014; Dasti et al., 2010; Humphrey et al., 2007).

Other animals are also common carriers of *Campylobacters* (Inglis et al., 2004; Hakkinen et al., 2007). Many studies have reported an association with cattle and sheep (Wesley et al., 2000; Nielsen, 2002; Stanley and Jones, 2003). Direct-contact exposure to bovine feces and ingestion of unpasteurized milk are well-documented causes of outbreaks of Campylobacteriosis (Bae et al., 2005).

Most *Campylobacter* infections in humans are self-limiting and do not require antimicrobial therapy. However, in systemic infections or in immunocompromised individuals, erythromycin and fluoroquinolones are used as the drugs of choice. Many studies have reported an increase in the resistance of *Campylobacter* to various antimicrobials, including the drugs of choice (Chen et al., 2010; Cody et al., 2010).

Development and transmission of antibiotic-resistant *Campylobacter* are complicated by the fact that *Campylobacter* is a zoonotic pathogen and is therefore exposed to antibiotics in animal production (Bogaard and Stobberingh, 2000; McEwen and Fedorka-Cray, 2002; Swartz, 2002).

In Algeria, there are few studies regarding Campylobacteriosis in humans (Guechi, 1984; Megraud et al., 1999) and food animals (El amir et al., 2013; Messad et al., 2013). Besides, no studies have been carried out on sheep and calves yet.

The present study was performed on food animals: sheep, calves and broilers in three slaughterhouses Bouira Province, central region of Algeria. Therefore, the objective of this study is: isolation and identification of *Campylobacter* germs, phenotypic characterization and the study of antibiotic resistance of the isolated strain.

MATERIALS AND METHODS

Samples collection

The study was conducted in two periods: the first from June 2009 to February 2010, and the second from August 2013 to January 2014.

The study targeted 200 sheep, 200 calves and 100 broilers randomly selected from a population of 1200 sheep, 2400 calves and 10 lots of broilers slaughtered in three rural slaughterhouses and a bird slaughterhouse located at Bouira Province.

Sheep samples were taken from different regions namely: Bouira, Oued Souf, Saida and Boumerdes. Avian and bovine samples come from the same region (Bouira).

Isolation and identification of *Campylobacter*

Stool samples were taken by rectal swab immediately after slaughter. The isolation in Petri dishes was performed on a Karmali medium, maximum one hour later *(Oxoid France; CM0935)* containing a selective supplement *(Oxoid France; SR0069E).*

The inoculated dishes were instantly placed in jars *(AnaeroJar Tm; Oxoid, AGOO25A)* containing Gas-pack bags for microaerophilic *(bioMérieux, France)* were used. The jars were incubated for 48 h at 37°C.

All bacterial colonies with a macroscopic appearance of *Campylobacter*, Gram stain and oxidase tests were performed. Strains were stored at - 80°C in BHIB (brain heart infusion broth) supplemented with 20% glycerol.

The strains belonging to the genus *Campylobacter* have been subjected to complete biochemical identification (catalase research by gallery species identification Api Campy *"bioMérieux, France"*) and an antibiotic sensitivity test.

Antimicrobial susceptibility testing of *Campylobacter* isolates

We tested antibiotics in the list of CA-SFM (CA-SFM, 2012), namely: Ampicillin (AM, 10 µg), amoxicillin + clavulanic acid (AMC, 10 µg), cephalothin (CF, 30 µg), cefotaxime (CTX, 15 µg), gentamicin (GM , 15 µg), tobramycin (TM, 10 µg), erythromycin (E, 15 UI), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TE, 30 UI), chloramphenicol (C, 30 µg).

Spiramycin (SP, 100 µg) and metronidazole (MTR, 16 µg) were added to the above list because of their use in veterinary medicine in Algeria.

The antimicrobial susceptibility test was performed by disk spray on Muller Hinton medium (Pasteur Institute of Algeria) with 5% blood with dishes incubation for 48 h at 37°C, in jars under a microaerophilic atmosphere at 37°C. In parallel, *Campylobacter jejuni* (ATCC 33560) was used as reference strain. The interpretation of the antibiogram was made according to the standards of CASFM (CA-SFM, 2012).

Data analysis

The results were analyzed using SPSS statistical software using the X2 test. Significant differences were considered when probability (p) was equal to or less than a risk ($p \leq 0.05$).

RESULTS

Prevalence of *Campylobacter* in sheep, calves and broiler chickens

Out of a total of 500 samples, 30% positivity rate (152/500) was observed. Depending on the species, the isolation rate was 13% (26/200), 14% (28/200) and 96% (96/100) for sheep, calves and broilers respectively (Table 1).

Campylobacter isolated species

In various animal species, we noted a predominance of the *C. jejuni* species with a frequency of 58% followed by *C. coli* (21%), *C. lari* (10%), *C. fetus* (7%) and *C. sputorum* (4%) (Figure 1).

The study of isolation frequency of the different *Campylobacter* species depending on the animal species showed that *C. jejuni* is the most common regardless of the animal species (Figure 2).

Table 1. *Campylobacter* isolation rates of different animal species (P <0.05).

Animal species	Number of samples	Number positives (%)
Sheep	200	26 (13%)
Calves	200	28 (14%)
Broiler chickens	100	96 (96%)
Total	500	150 (41%)

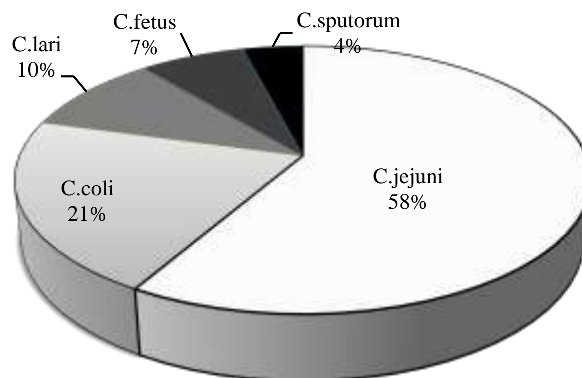

Figure 1. Total frequency of isolation of different species of *Campylobacter*.

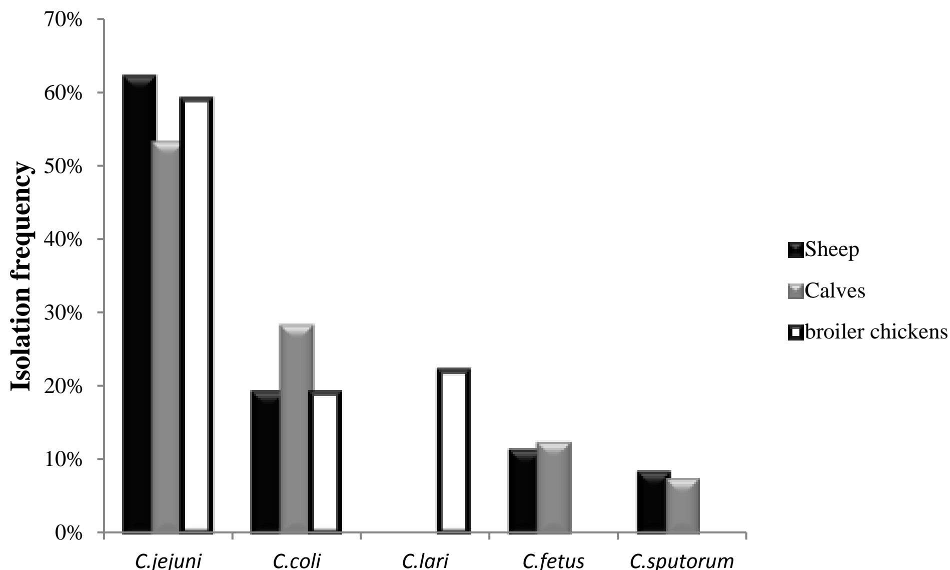

Figure 2. *Campylobacter* species isolated in different animal species.

Isolation rate depending on the region of sheep origin

In sheep, the isolation rate of *Campylobacter* strains differs depending on the regional origin of animals. Indeed, a significant difference was recorded between the positivity rates of the different regions (Table 2).

Antimicrobial susceptibility

The strains isolated from the broiler chickens had

Table 2. Isolation rate strains in sheep by region.

Parameter	Region				Total N=200
	Oued Souf (n=50)	Bouira (n=50)	Saida (n=50)	Boumerdes (n=50)	
Number of isolated strains	7	7	9	3	26
Percentage of positives	26.92	26.92	34.62	11.54	13

(P_1-value =0,000<0,05). P_1-value: Value for the isolation rate of the sheep's strains isolated from different regions.

Table 3. *Campylobacter* resistance rates among different animal species.

Antibiotic	Sheep (N=26)	Calves (N=28)	Broiler chickens (N=98)	P_2-Value
	n (%)	n (%)	n (%)	
AM	9 (34.62%)	13 (50.00%)	78 (81.25%)	P<0.05
AMC	4 (15.38%)	3 (11.54%)	72 (75.00%)	P<0.05
CF	8 (30.77%)	6 (23.08%)	49 (51.04%)	P<0.05
CTX	3 (11.54%)	9 (34.62%)	38 (39.58%)	P<0.05
GM	8 (30.77%)	7 (26.92%)	45 (46.88%)	P<0.05
K	4 (15.38%)	2 (7.69%)	10 (10.42%)	P<0.05
TM	4 (15.38%)	2 (7.69%)	10 (10.42%)	P<0.05
E	15 (57.69%)	10 (38.46%)	85 (88.54%)	P<0.05
SP	10 (38.46%)	16 (61.54%)	35 (36.46%)	P<0.05
TE	11 (42.31%)	6 (23.08%)	43 (44.79%)	P<0.05
C	8 (30.77%)	2 (7.69%)	10 (10.42%)	P<0.05
MTR	16 (61.54%)	27 (85%)	86 (89.58%)	P<0.05
AN	16 (61.54%)	2 (7.69%)	93 (96.88%)	P<0.05
CIP	1 (3.85%)	2 (7.69%)	88 (91.67%)	P<0.05

N: Number of strains, n: Number of strains resistance to antibiotic. P_2-Value: Value for the antimicrobial resistance difference between the strains isolated from sheep, calves content and those isolated from broiler chickens samples to the same antibiotic.

significantly greater rates of resistance compared to *Campylobacter* strains isolated in sheep and calves.

The highest levels of resistance broiler strains were noted to ciprofloxacin (91.6%), nalidixic acid (96.8%) and Erythromycin (88.5%).

In this study, different strains isolated had varying levels of resistance depending on the antibiotic and animal species (Table 3).

DISCUSSION

A variation in the prevalence of *Campylobacter* was found between the different studies in different parts of the world in sheep, cattle (Stanley and Jones, 2003) and in broilers (Jorgensen et al., 2011).

In our present work, we noted 26 and 28% positivity rates for sheep and calves, respectively, and 96% positivity rate for broilers. This result is consistent with some studies (Goualie et al., 2005; Dadi et al., 2008; Salihu et al., 2009a, b; Messad et al., 2013) but is different from others (Desmonts et al., 2004; Igimi et al., 2008; Bae et al., 2005; Garcia et al., 2010).

According to certain authors, the variation in *Campylobacter* isolation frequency observed between different studies would be linked to a number of factors such as: the season (Hannon et al., 2009), geographic location (Berrang et al., 2000) as well as the sample size (Jeffrey et al., 2001).

The choice of research method and use of enrichment media can also play an important role. Indeed, Garcia et al. (2010) and Hakkinen et al. (2007) showed an improvement of 15 and 30% respectively due to the use of a medium enrichment.

In various animal species, we noted a prevalence of *C. jejuni* (62, 53 and 59% in sheep, calves and broiler respectively), which is consistent with most studies in the world. Such as 79.6% for Nigeria according to Salihu et al. (2009a), 79.6% for China according to Chen et al. (2010), 47.7% for Italy according to Parisi et al. (2007), 87 and 34.1% for Canada according to Hakkinen et al. (2007) and Hannon et al. (2009), respectively. The reasons for the variations are unknown and could be attributable to differences in production practices and

environments (Chen et al., 2010).

From the viewpoint of sensitivity to antibiotics, *Campylobacter* strains isolated from the broiler had significantly higher rates of resistance compared to the ovine and bovine strains.

It is noteworthy that several studies which have dealt with the antibiotic susceptibility of *Campylobacter* strains isolated from broiler samples reported similar rates in our vis-à-vis ciprofloxacin (91.6%) (Kang et al., 2006; Chen et al., 2010; Kovalenko et al., 2014) and nalidixic acid (96.8%) (Kang et al., 2006; Rahimi et al., 2010; Chen et al., 2010; Kovalenko et al., 2014).

The high fluoroquinolone-resistance rates of *Campylobacter* may be attributed to the widespread use of fluoroquinolones in poultry production in Algeria. This class of antibiotics is used for both prevention and control of poultry diseases. It is well known that the use of fluoroquinolones in poultry selects fluoroquinolone resistant mutants and leads to the emergence of fluoroquinolone-resistant *Campylobacter* in the treated birds.

Teuber suggested that the use of enrofloxacin (derivates close to the fluoroquinolones used in human medicine) in animals flocks has probably exerted a selection pressure in animal reservoirs (Teuber, 2001).

About Erythromycin, we noted in our study a high level of resistance to this antibiotic in broilers (88.5%). According to a WHO (2008) report, macrolides are widely used in farms, and this practice is known to promote the selection of resistant *Campylobacter* strains in animals. Lin et al showed that the use of Erythromycin in low dose for a long time selects *Campylobacter* strains resistant to this antibiotic (Lin et al., 2007).

In this study, a high proportion of *Campylobacter* avian resisted to metronidazole (89%). According to the study of Stanley and Jones (2003), already 80 to 100% of *Campylobacter* strains were resistant to this antibiotic in 1998.

About tetracycline, the resistance rate (44.7%) we got for all avian strains is much higher than reported by Kuana et al. (2008) in Brazil that varies between 15 to 16% and by Ronner et al. noted in a study in four European countries (35.4%). This rate is similar to the rate of resistance noted by Beatriz et al. in Spain (Oporto et al., 2009).

In our series of *Campylobacter* strains, low percentages of resistance to chloramphenicol were found, in accordance with the results presented in previous publications conducted in Algeria (Messad et al., 2013). High susceptibility to chloramphenicol could be explained by none or moderate use of this antibiotic due to its non-registration in Algeria since 2006 (WHO, 2008).

Conclusion

The prevalence of *Campylobacter* in calves and sheep slaughter was low, indicating that calves and sheep can be considered as a minor source of *Campylobacter* infection to consumers.

Unlike that of broilers, which showed a high isolation rate (96%). High levels of resistance to certain antibiotics in strains isolated from different animal species, and multi detected resistance can be explained by the use of these antimicrobial agents in animals. Therefore, proper strategies have to be designed and implemented to minimize its effect on broilers, and also calves and sheep as well, besides this, correct drug usage should be recommended to minimize resistance so as to increase livestock production in the studied district.

Conflict of interests

The authors have not declared any conflict of interest.

REFERENCES

Bae W, Kaya KN, Hancock DD, Call DR, Park YH, Bessel TE (2005). Prevalence and antimicrobial resistance of thermophilic Campylobacter spp. From cattle farms in Washington state. Appl. Environ. Microbiol. 71:169-174.

Berrang ME, Buhr RJ, Cason JA (2000). Campylobacter Recovery from external and internal of commercial broilers carcass prior to scalding. Poult. Sci. 79(2):286-290.

Bogaard VD, Stoberingh EE (2000). Epidemiology of resistance to antibiotic links between animals and humans. Int. J. Antimicrob. Agents 14(4):327-35.

Chen X, Naren GW, Wu C, Wang M, Dai Y, Xia L (2010). Prevalence and antimicrobial resistance of Campylobacter isolates in broilers from China. Vet. Microbiol. 144:133-139.

Cody AJ, Colles FM, Sheppard SK, and Maiden MCJ (2010). Where does Campylobacter come from? A molecular odyssey. Adv. Exp. Med. Biol. 659:47-56.

Dadi L, Astrat D (2008). Prevalence and antimicrobial susceptibility profiles of Thermotolerant Campylobacter strains in retail raw meat production Ethiopia. Ethiop. J. Heath Dev. 22(2):195-200.

Dasti JI, Tareen AM, Lugert R, Zautner AE, Gros U (2010). Campylobacter jejuni: A brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int. J. Med. Microbiol. 300:205-211.

Desmonts MH, Dufour-Gesbert F, Avrain L, Kempf I (2004). Antimicrobial resistance in Campylobacter strains isolated from French broilers before and after antimicrobial growth promoter bans.J Antimicrob Chemother. 54(6):1025-1030.

EL Amir L, Mouffok F., Hellal A (2013). Campylobacter in poultry research in Algeria: Study of antibiotic resistance profile. Med J. Vet 164(6):307-311.

Garcia AB, Steele WB, Taylor DJ (2010). Prevalence and carcass contamination with Campylobacter in sheep sent for slaughter in Scotland. J. Food Saf. 30:237-250.

Goualie GB, Karou GT, Bakayoko S, Coulibaly K, Coulibaly KE, Niamke S L and Dosso M (2005). Prevalence of Campylobacter in chickens sold in the markets of Abidjan: Pilot study in the common Adjamé in 2005. Afr. J. Health Anim. Prod. RASPA Vol.8.

Guechi Z (1984).*Campylobacter jejuni* etiologic agent of diarrhea in Algeria. Preliminary data. Diarrhea young. Colloque, Pp. 341-344 (7 ref).

Hakkinen M, Heiska H, Hänninen ML (2007). Prevalence of Campylobacter spp. in cattle in Finland and antimicrobial susceptibilities of bovine Campylobacter jejuni strains. Appl. Environ. Microbiol. 73(10):3232-3238.

Hannon SJ, Allan B, Waldner C, Russell ML, Potter A, Babiuk LA, Townsend HGG (2009). Prevalence and risk factor investigation of *Campylobacter* species in beef cattle feces from seven large

commercial feedlots in Alberta, Canada. Can. J. Vet. Res. 73(4):275-282.

Humphrey T, O'Brien S, Madsen M (2007). Campylobacters as zoonotic pathogens: a food production perspective. Int. J. Food Microbiol. 117:237-257.

Igimi S, Okada Y, Ishiwa A, Yamasaki M, Morisaki N, Kubo Y, Asakura H, Yamamoto S (2008). Antimicrobial resistance of Campylobacter: Prevalence and trends in Japan. Food Addit. Contam. 25(9):1080-103.

Inglis GD, Kalischuk LD, Busz HW (2004). Chronic shedding of *Campylobacter* species in beef cattle. J. Appl. Microbiol. 97:410-420.

Jeffrey JS, Tonooka KH, Lozanot J (2001). Prevalence of campylobacter spp. from skin, crop, and intestine of commercial broiler chicken carcasses at processing. Poult. Sci. 80(9):1390-1392.

Jorgensen F, Ellis-Iversen J, Rushton S, Bull SA, Harris SA, Bryan SJ, Gonzalez A, Humphrey TJ (2011). Influence of season and geography on Campylobacter jejuni and C. coli subtypes in housed broiler flocks reared in Great Britain. Appl. Environ. Microbiol. 77(11):3741-3748.

Kang YS, Cho YS, Yoon SK, Yu MA, Kim CM, Lee JO, and Pyun YR (2006). Prevalence and antimicrobial resistance of Campylobacter jejuni and Campylobacter coli isolated from raw chicken meat and human stools in Korea. J. Food Prot. 69:2915-2923.

Kovalenko K, Roasto M, SantareS, Berzi A, Hörman A (2014). Campylobacter species and their antimicrobial resistance in Latvian broiler chicken production. Food Control 46:86-90.

Kuana SL, Santos LR, Rodrigues LB, Borsoi A, Moraes HL, Salle CT, Nascimento VP(2008). Occurrence and characterization of Campylobacter in the Brazilian production and processing of broilers. Avian Dis. 52(4):680-684.

Lin J, Yan M, Sahin O, Pereira S, Chang Y J, Zhang Q (2007). Effect of macrolide usage on emergence of erythromycin resistant Campylobacter isolates in chickens. Antimicrob. Agents Chemother. 51(5):1678-1686.

Lupo C, Le Bouquin S, Bougeard S, Balaine L, Gilli-Dunoye P, Hérau V, Michel V, Péraste J, Petetin I, Colin P, Chauvin C (2014). An epidemiological approach to health inspection of poultry to slaughter, Epidemiological Bulletin, Animal Health and Food No. 41.

McEwen SA, Fedorka-Cray PJ (2002). Antimicrobial use and resistance in animals. Clin. Infect. Dis. 34(Supplement 3):S93-106.

Megraud F, Boudraa G, Bessaoud K, Bensid S, Dabis F, Soltana R, Touhami M (1990). Incidence of Campylobacter infection in infants in western Algeria and the possible protective role of breast feeding. Epidemiol. Infect. 105(01):73-78.

Messad S, Hamdi TM, Bouhamed R, Ramdani-Bouguessa N, Tazir M (2013). Frequency of contamination and antimicrobial resistance of thermotolerant Campylobacter isolated from some broiler farms and slaughterhouses in the region of Algiers. Food Control 40:324-328.

Nielsen EM (2002). Occurrence and strain diversity of thermophilic campylobacters in cattle of different age groups in dairy herds. Lett. Appl. Microbiol. 35:85-89.

OIE : Terrestrial Manual of the International Organization for Animal Health (2008). *Campylobacter jejuni* and *Campylobacter coli*, Chapter 2/09/3. P 1185.

Oporto B, Juste RA, Hurtado A (2009). Phenotypic and Genotypic Antimicrobial Resistance Profiles of *Campylobacter jejuni* Isolated from Cattle, Sheep, and Free-Range Poultry Faeces. Int. J. Microbiol. Volume 2009, Article ID 456573. 8 p.

Parisi A, Lanzilotta SG, Addante N, Normanno G, Di Modugno G, Dambrosio A, Montagna CO (2007). Prevalence, molecular characterization and antimicrobial resistance of thermophilic *Campylobacter* isolates from cattle, hens, broilers and broiler meat in south-eastern Italy. Vet. Res. Commun. 31(1):113-123.

Rahimi E, Momtaz H, Ameri M, Ghasemian-Safaei H, Ali-Kasemi M (2010). Prevalence and antimicrobial resistance of *Campylobacter* species isolated from chicken carcasses during processing in Iran. Poult. Sci. 89(5):1015-1020.

Salihu MD, Abdulkadir JU, Oboegbulem SI, Egwu GO, Magaji AA, Lawal M, Hassan Y (2009a). Isolation and prevalence of *Campylobacter* species in cattle from Sokoto State, Nigeria. Vet. Ital. 45:501-505.

Salihu MD, Junaidu AU, Oboegbulem SI, Egwu GO (2009b). Prevalence and Biotypes of *Campylobacter* Species Isolated from Sheep in Sokoto State, Nigeria. Int. J. Anim. Vet. Adv. 1(1):6-9.

Stanley K, Jones K (2003). Cattle and sheep farms as reservoirs of *Campylobacter*. J. Appl. Microbiol. 94:104S-113S.

Swartz MN (2002). Humain diseases caused by foodborne pathogens of animal origin. Clin. Infect. Dis. 34(Suppl 3):S111-S122.

Teuber M (2001). Veterinary use and antibiotic resistance. Curr. Opin. Microbiol. 4:493-499.

Wesley IV, Wells' S J, Harmon K M, Green A, Schroeder-Tucker L, Glover M, Siddique I (2000). Fecal shedding of *Campylobacter* and *Arcobacter spp.* in dairy cattle. Appl. Environ. Microbiol. 66:1994-2000.

WHO- World Health Organisation (2008). Antimicrobial resistance from animals for food. INFOSAN Information Note No. 2/2008.

WHO-World Health Organization (2012). The global view of Campylobacteriosis: report of an expert consultation, Food Safety, Utrecht Netherlands, ISBN 978 92 4 156460 1. P 69.

academicJournals

Vol. 10(39), pp. 1668-1674, 21 October, 2016
DOI: 10.5897/AJMR2016.8266
Article Number: C4438FD61255
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effects of immunoglobulin Y (IgY) serum against plaque bacteria

Juni Handajani[1]*, Dhinintya Hyta Narissi[2], Mufidana Azis[2] and Aurita Siwi Rahmawati[2]

[1]Oral Biology Department, Faculty of Dentistry, Universitas Gadjah Mada, Indonesia.
[2]Dentistry Major, Faculty of Dentistry, Universitas Gadjah Mada, Indonesia.

Received 11 August 2016, Accepted 10 October, 2016

Dental caries is an infectious disease caused by plaque bacteria, especially *Streptoccus mutans*. Many studies were conducted to develop methods or material for dental caries prevention. It was using passive immunization that will produce specific antibodies. Yolk immunoglobulin (IgY) is antibody in the serum and egg yolk. This study aimed to determine the effect of IgY serum against the growth of dental plaque bacterial in vitro. In the long term, it is expected IgY specific *S. mutans* can be as one of material could prevent dental caries topically. Immunization in the chicken was performed using *S. mutans* antigen. Immunoglobulin Y (IgY) specific *S. mutans* was collected from blood serum of Hysex Brown chicken. Antibacterial effect of IgY serum on the growth of bacteria that cause dental plaque (*Streptococcus alpha, Staphylococcus aureus,* and *S. mutans*) was done using diffusion method. Sample was performed on 10 plate each bacteria. Data inhibition zone were analyzed using ANOVA and LSD. There was no inhibition zone of IgY serum on the growth of *S. alpha* and *S. aureus* while positive results obtained against *S. mutans*. Inhibition zone increased according increasing the concentration of IgY. ANOVA test showed significant differences the effect of concentration IgY serum on the inhibition zones of *S. mutans* (p <0.05). A significant difference (p<0.05) from the LSD was obtained in the comparison between the concentration of IgY, IgY serum and controls. In conclusion, IgY serum that immunized *S. mutans* has antibacterial effect against *S. mutans* only.

Key words: *Yolk immunoglobulin* (IgY), chicken serum, *Streptococcus alpha, Staphylococcus aureus, Streptococcus mutans*

INTRODUCTION

Dental caries is an infectious disease which can be found in almost all people in the world. Dental caries is known as a multifactorial disease. *Streptoccus mutans* is known as dental caries bacteria. This disease has been known since the nineteenth century, from the textbook Black noted that dental caries is defined simply as the chemical dissolution of calcium in teeth by lactic acid, followed by decomposition of the organic matrix of dentin.

*Corresponding author. E-mail: junihandajani@ugm.ac.id.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

In the mid-nineteenth century,caries observed in Europeans was severe. At that time a lot of people aged around 14 year old had lost teeth due to dental caries which incriminate pulp (Fejerskov and Kidd, 2003).

Until now, dental caries condition is still quite alarming, it can be seen from the result of the Household Health Survey (SKRT) in 2015 (Ministry of Health, 2015) that the prevalence of caries disease in Indonesia is still showing significant number which is 90.05%. Problems which is still occurring now is whether dental caries as an infectious disease can be prevented or not. Fejerskov and Kidd (2003) expressed their opinion that the formation of caries cavity can be prevented through the control of process which involving caries however metabolic fluctuations in biofilms cannot be prevented. Various efforts to prevent the formation of dental caries cavity have been performed, such as flour application in drinking water as well as in toothpaste. Also immunization against dental caries though it is still in the research stage.

One of the attempts to immunize against dental caries is producing antibodies that will be expected to be able to prevent caries cavity. Yolk Immunoglobulin (IgY) is immunoglobulin found in chicken egg which is derived from the blood as a result of exposure to antigen. The use of specific IgY is known to be beneficial for treatments or therapy. Hatta et al. (1990) have found a simple method for the isolation of yolk antibodies. The production of specific IgY against *S. mutans* through passive immunization has been carried out by Hatta et al. (1994) and Poetri and Soejoedono (2006).

Normal flora in the oral cavity are known to be complex and about 350 species of bacteria have been cultured, however still a lot of species of bacteria that are still in the stage of identification. *Streptococcus* genus in the oral cavity consists of 4 main species which are *mutans, salivarius, anginosus* and *mythic. Streptococcus* can be classified according to degrees of hemolysis on blood agar (Samaranayake, 2001).

Dental plaque is composed by micro-organisms, especially bacteria. One gram plaques are composed of about $2x10^{11}$ bacteria. Early bacteria colonization on the tooth surface occurs and within a few hours the bacteria can already be found on the pellicle. The bacteria that hold the initial colonization of the pellicle on the surface of the oral tissues is especially facultative Gram-positive bacteria such as *Actinomyces viscosus* and *Streptococcus sanguinis* (Newman et al., 2002). According to Michalek and Mc Ghee (1982) *Streptococcus alpha* or *Streptococcus viridans* were also known as the dominant bacteria in early plaque formation and this bacteria is to facilitate the colonization of other bacteria including anaerobic bacteria which has a big role in periodontal disease. In addition, *Streptococcus* also produces histolytic enzymes and toxic substances that can damage tissues. Growth of bacteria in the oral cavity is important to know because the disease originated from the change of bacteria from normal flora to a pathogenic bacteria. Preliminary research on the effectiveness of egg yolk antibodies (IgY) as an anti-caries ingredient through the hydrophobicity test against *S. mutans* has been done by Azis et al. (2013) through Research - Student Creativity Program (PKM-P). Mechanism of IgY antibodies against bacterial growth is important to be learned because it is expected to be a material that is able to maintain homeostasis of microflora in the mouth so that in the long-term can be used as a preventive to prevent dental caries. Also about the effects of yolk immunoglobulin (IgY) serum on the growth of bacteria of dental plaque bacteria *in vitro* (Studies on Chicken Serum which is immunized by *Streptococcus mutans*). Therefore, this study aimed to determine the effects of yolk immunoglobulin (IgY) serum towards the growth of dental plaque bacteria in vitro so that in the long term it is expected to be used as prevention of dental caries.

MATERIALS AND METHODS

This study was a pure laboratory experimentation. The procedure of this study was approved by the Ethics Committee, Faculty of Dentistry, Universitas Gadjah Mada. The study was conducted at the Laboratory of Microbiology, Faculty of Veterinary, Universitas Gadjah Mada.

Immunization of experimental animals

Four Hysex Brown chickens aged 18-24 weeks were immunized with 0.5 ml (10^9 CFU) suspension of *S. mutans* intravenously for three consecutive days. Followed by injection of 1 ml (10^9 CFU) suspension of bacteria into complete Freund's adjuvant at week 2, and 1 ml (10^9 CFU) suspension of bacteria *S. mutans* into incomplete Freund's adjuvant at week 3 and 4 intramuscularly. After 1 week serum was collected.

Difussion test

S. alpha and *Staphylococcus aureus* were from stock bacteria in the Laboratory of Microbiology, Faculty of Veterinary, Universitas Gadjah Mada. *S. mutans* ATCC 25175 was procured from Dr. Yulita Kristanti, Department Conservative Dentistry, Universitas Gadjah Mada. *S. mutans* has been subcultured and re-identified in the Laboratory of Microbiology, Faculty of Medicine, Universitas Gadjah Mada.

S. alpha, S. aureus, and *S. mutans* were cultured on blood plates media then incubated for 18-24 h at 37°C. Colony of bacteria was taken from the subculture media and then put into 3 ml of liquid Brain Heart Infusion (BHI) media and incubated for 18 hours in an incubator at 37°C. The resulting bacterial suspension in BHI media was compared to the turbidity of 0.5 McFarland standard which is equivalent to 10^8 CFU/ml. Disk diffusion test were done by measuring the diameter of the clear zone as an indication of bacterial growth inhibitory response by an antibacterial compound.
Bacterial growth was observed to see the formation of inhibition zones around the pits. Diameter of inhibition zones that was clear

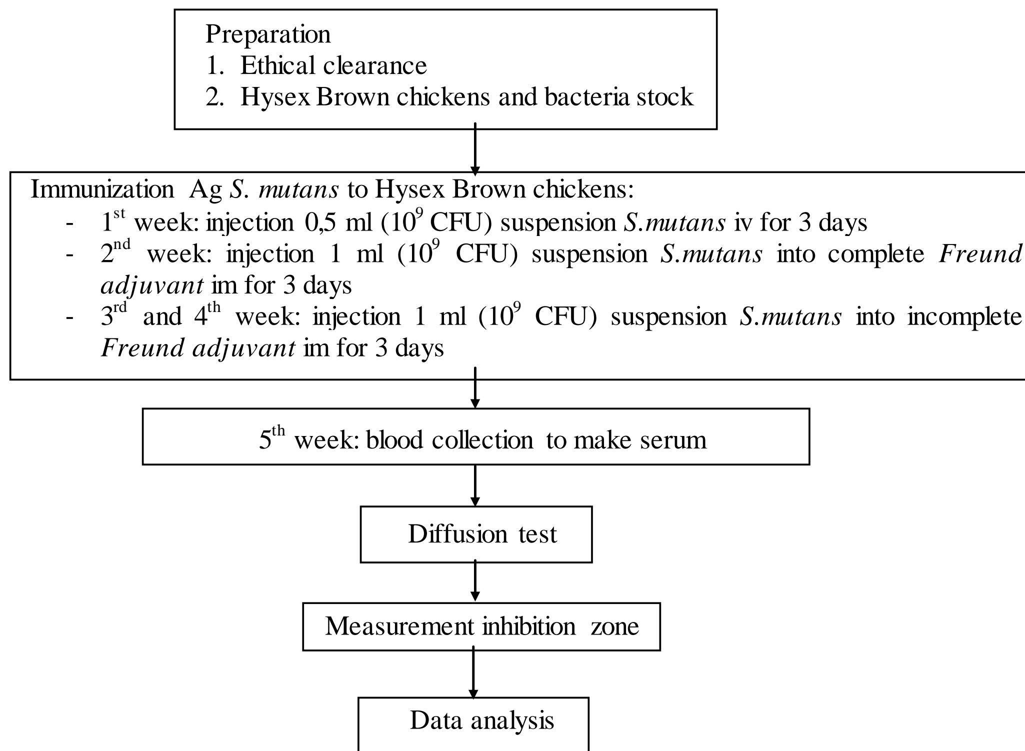

Figure 1. Flowchart of study.

area (there was no bacterial growth) around the wells were measured using a caliper.

Statistical analysis

All data were analyzed using normality test and homogeneity of variance test, then analyzed using statistical tests Analysis of Variance (ANOVA) and post hoc Least Significant Difference (LSD) (Figure 1).

RESULTS

Positive result of IgY was detected using Agar Gel Precipitation Test (AGPT), a positive reaction was marked by the precipitation line between the antigen pit and antibody. Azis et al. (2013) resulted that the positive result of IgY in chicken egg yolk were obtained at week 5 after immunization so that the study was conducted after taking the serum at week 5 after immunization. IgY antibacterial activity testing of some IgY serum concentration levels that have been immunized with *S. mutans* were done against the dental plaque bacteria. The test results of IgY against bacteria *S. alpha* and *S. aureus* were negative or did not form inhibition zones (Figure 2).

The ability of antibacterial activity in some IgY serum concentration immunized with *S. mutans* was obtained against *S. mutans.* The test results of Minimal Inhibitory Concentration (MIC) and inhibition zones of several concentrations of IgY against *S. mutans* were shown in Figure 3.

Measurement were done on the inhibition zone on 10 plate. In Figure 3 it can be seen that the minimum concentration of IgY *S. mutans* spesific that has inhibition against *S. mutans* was a concentration of 25%. Highest inhibition zone were obtained on the positive control (chlorhexidine digluconate). On the solvent control or a negative control using distilled water did not form visible inhibition zone. Mean result (n = 10) and standard deviation inhibition zone several concentrations of IgY *S. mutans* spesific and control of *S. mutans* shown in Figure 4.

The Kolmogorov-Smirnov was used for normality calculation because the amount of data is 50. The result of the normality test showed $p > 0.05$ or normal data distribution. Furthermore, homogeneity test showed $p > 0.05$ or can be interpreted as homogeneous data. ANOVA was conducted to determine differences in inhibition zone of some concentrations of IgY serum *S. mutans* specific against *S. mutans* (Table 1).

Table 1 show that there was significant difference in

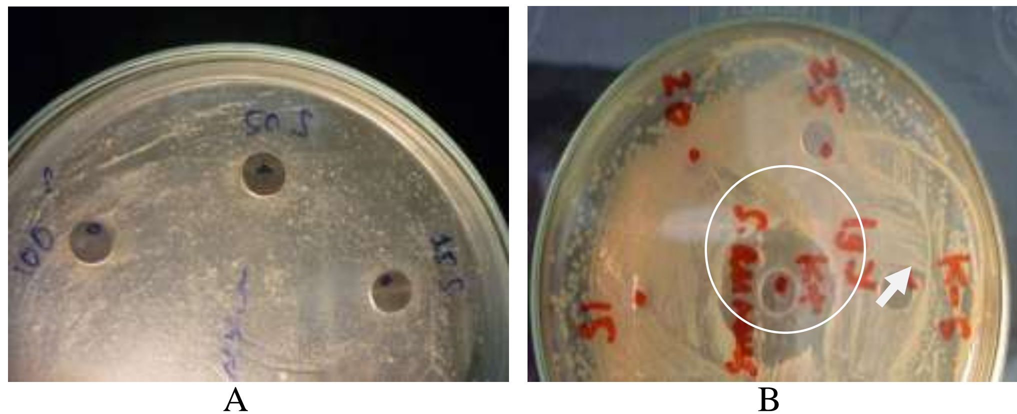

Figure 2. There was no inhibition zone the antibacterial activity of IgY serum against *S. alpha* (A) and *S. aureus* (B) by diffusion test. Inhibition zone formed on the positive control using chlorhexidine digluconate (circles) but not formed on the negative control using distilled water.

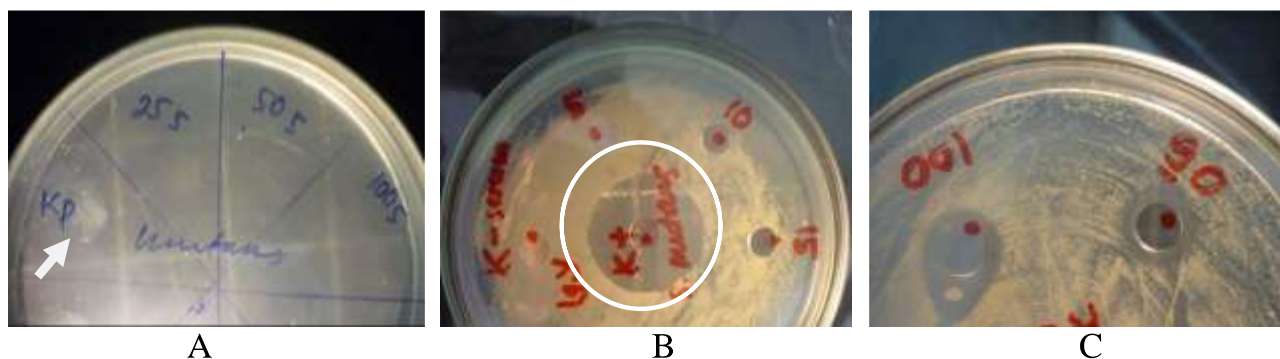

Figure 3. Antibacterial activity of IgY serum that has been immunized against *S. mutans.* The minimum concentration showed at 25% IgY against *S. mutans* (A). Inhibition zone was formed at a concentration of 50% and 100% (C) and a positive control (B) using chlorhexidine digluconate (circles) but not formed on the solvent control or negative control using distilled water (arrows).

inhibition zones formed from several concentration of IgY *S. mutans* specific and control of *S. mutans.* Statistical testing was continued using LSD to compare each concentration as well as the control (Table 2).

In Table 2, it can be seen that all comparisons between treatment groups either concentrations of IgY serum *S. mutans* specific or IgY serum with control indicating $p < 0.05$. The results showed a significant difference between the ratio of concentrations of IgY serum *S. mutans* specific and control of the inhibition zone formed in *S. mutans.*

DISCUSSION

The microorganisms found in the human oral cavity have been referred to as the oral microflora, oral microbiota, or more recently as the oral microbiome. Approximately, 280 bacterial species from the oral cavity have been isolated in culture and formally named. It has been estimated that less than half of the bacterial species present in the oral cavity can be cultivated using anaerobic microbiological methods and that there are likely 500 to 700 common oral species (Saini, 2015). The results showed immunization using antigen *S. mutans* for 4 weeks will result an antibody IgY serum specific *S. mutans* in the 5th week. It was aligned with previous study that IgY serum specific *S. mutans* in chicken egg yolk using Agar Gel Precipitation Test (AGPT) generated at week 5 (Azis et al., 2013). Bizanov and Jonauskiene (2003) also reported that after 2 weeks of initiation immunization, high immunospecific antibodies will be generated in the chicken egg yolk and that antibody titer will increase until the 7th week. Immunospecific antibodies will be actively transported from the serum to the chicken egg yolk during chicken pregnancy which immunized with

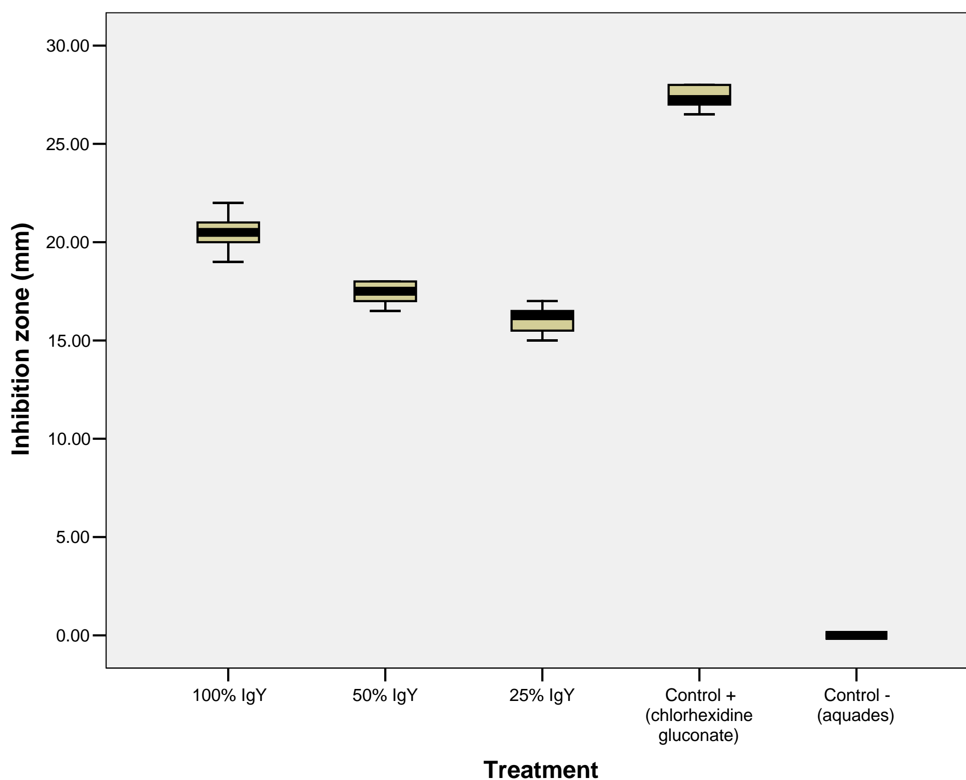

Figure 4. The mean inhibition zone of some concentrations IgY serum specific to S. mutans and control (n = 10). The highest results of inhibition zone (mm) were seen in the positive control. IgY serum inhibition zone increased along with increasing concentrations.

Table 1. ANOVA results showed inhibition zone some concentrations of IgY serum specific *S. mutans*.

Zone	Sum of squares	df	Mean square	F	Sig.
Between Groups	4065.850	4	1016.463	2647.804	0.000
Within Groups	17.275	45	0.384		
Total	4083.125	49			

Table 2. Summary of LSD test showed comparison inhibition zone (mm) between several concentrations of IgY serum specific *S. mutans* and control.

Concentration	25% IgY	50% IgY	100% IgY	Control +	Control -
25% IgY	-	0,000	0,000	0,000	0,000
50% IgY	0,000	-	0,000	0,000	0,000
100% IgY	0,000	0,000	-	0,000	0,000
control +	0,000	0,000	0,000	-	0,000
control -	0,000	0,000	0,000	0,000	-

specific antigens. Antibody specific *S. mutans* in chicken serum which was detected using ELISA appears first in 7 days after immunization and then detected in chicken egg yolk after 2 weeks. Specific antibodies titer will increase

in the chicken serum and reach the peak on day 45 and will remain stable until day 168 (Rajan et al., 2011). The results support previous study that the resulting IgY serum is specific to a particular immunized antigen (Rajan et al., 2011; Dinesh et al., 2013). It can be shown in Figure 2 that the IgY serum only has antibacterial activity against *S. mutans* and not against *S. aureus* and *S. alpha. S. mutans* is referred as the causative agent of caries through three stages, beginning with interactions with tooth surface mediated by adhesion, accumulation of bacteria in biofilm and the production of glucose and glucan by glucosyltransferase enzyme of bacteria, as well as the formation of lactic acid. Glucosyltransferase enzyme in bacteria is able to synthesize extracellular polysaccharides, especially hydrophobic glucan from sucrose and its ability to colonize with the tooth surfaces (Koo et al., 2010).

Figures 2 and 3 show the IgY serum *S. mutans* specific has antibacterial activity against *S. mutans.* Mechanism of antibacterial activity of the IgY *S. mutans* specific is suspected because of the specific IgY serum can weaken the pathogenicity of *S. mutans.* This is consistent with previous studies that IgY *S. mutans* specific could be expected to prevent dental caries by means of bacteria immobilization and demolish the bacteria's ability to convert sugar into acid. Prevention of adhesion of *S. mutans* to tooth surfaces can be done by using an antibody that has a target to block an antigenic adhesion of pathogens (Rajan et al., 2011). The ability of IgY specific to bind to the pathogenic bacteria will change the bacteria surface that can weaken the bacteria to attach (adhere) to the host cell. Hamajima et al. (2007) mentions that specific IgY can bind to the outer membrane proteins (outer membrane protein / OMP) of *P. gingivalis* bacteria that inhibit these bacteria to conduct co-aggregation. Other mechanism mentioned that a high IgY antibody titer will have great purity and effectively to neutralize various antigens *in vitro* and *in vivo*. As noted in the study of Meenatchisundaram et al. (2008) that IgY egg yolk has the ability to neutralize toxins from cobra. Other study mentions that IgY *S. aureus* specific given as an infusion into the mammary gland of cattle could inhibit the growth of *S. aureus* as a cause of mastitis.

Positive control using mouthwash ingredient chlorhexidine digluconate (CHX) showed antibacterial activity against *S. mutans, S. aureus* and *S. alpha* higher than IgY serum treatment. This is due to differences in the mechanism of antibacterial activity of both ingredients. IgY serum *S. mutans* specific has neutralization mechanism or weaken the ability of bacteria by binding to the bacteria and does not have the ability to kill bacteria while CHX has a toxic ability towards the cell despite of the low toxicity. In addition CHX containing bis-biguanide biocide cationic is known to have broad spectrum of antibacterial effect which causing disruption of bacterial cell membranes and death of cells. CHX cationic nature allows it to bind to the tooth surface and oral mucosa that inhibits the formation of dental plaque and prevent gingivitis. Rinse using CHX for 5 days can reduce the number of *S. mutans* between 30-50% and the repeated use of mouthwash that can lead to changes in the oral flora ecosystem (McBain et al., 2003).

Distilled water is used for negative control or solvent in this study. The result shows the negative control has no antibacterial activity at all three bacteria tested. It can be concluded that the immunization of *S. mutans* produces IgY specific *S. mutans* serum and IgY specific *S. mutans* serum has antibacterial activity against *S. mutans* only.

Conflict of interest

The authors declare that they have no conflict of interest

ACKNOWLEDGEMENT

This study was supported by Grant (Dana Masyarakat) from Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

REFERENCES

Azis M, Narissi DH, Siwi AR, Dwi KM, Dias N, Handajani J(2013). The time required to produce IgY chicken egg yolk immunized with *S. mutans*. MKG 20(1):31-34.

Bizanov G, Jonauskiene I (2003). Production and Purification of IgY from Egg Yolk after immunization of henz with PIG IgG, Bull. Vet Inst Pulawy 47:403-10.

Dinesh MD, Uma M.S., Anjali VM, Shanmugam V, Meenatchisundaram S, Michael A (2013). Characterization and in vitro neutralization of *Streptococcus mutans* egg yolk antibodies (IgY). Int. J. Pharm. Pharm Sci. 5(2):258-262.

Fejerskov O, Kidd E (2003). Dental Caries: The Disease and its Clinical Management, Australia: Blackwell Munksgaard. Pp.29-32, p38, Pp.168-171, p186 and p235

Hamajima S, Maruyama M, Hijiya T, Hatta H, Abiko Y (2007). Egg yolk-derived immunoglobulin (IgY) against *Porphyromonas gingivalis* 40-kDa outer membrane protein inhibits coaggregation activity. Arch. Oral Biol. 52(7):697-704.

Hatta H, Kim M, Yamamoto T (1990). A novel isolation method for hen egg yolk antibody, "IgY", Agric. Biol. Chem. 54(10):2531-2535.

Hatta H, Tsuda K, Ozeki M, Kim M, Yamamoto T, Otake S, Hirasawa M, Katz J, Childers NK, Michalek SM (1997). Passive immunization against dental plaque formation in humans: effect of a mouth rinse containing egg yolk antibodies (IgY) specific to *Streptococcus mutans*. Caries Res. 31:268-274.

Koo H, Xiao J, Klein MI, Jeon JG (2010). Exopolysaccharides Produced by *Streptococcus mutans* Glucosyltransferases Modulate the Establishment of Microcolonies within Multispecies Biofilms. J. Bacteriol. 192(12):3024-3032.

McBain AJ. Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Gilbert P (2003). Effects of a Chlorhexidine Gluconate-Containing Mouthwash on the Vitality and Antimicrobial Susceptibility of *in vitro* oral bacterial ecosystems. Appl. Environ. Microbiol. 69(8):4770-4776.

Meenatchisundaram S, Parameswari G, Michael A, Ramalingam S (2008). Neutralization of the pharmacological effects of cobra and krait venom by chicken egg yolk antibodies. Toxicon 52: 221-227.

Michalek SM, Mc Ghee JR (1982). *Streptococcus* with Emphasis on

Streptococcus mutans in Mc Ghee JR, Dental Microbiology, Philadelphia: Harper and Row Publisher. pp. 685-686.
Ministy of Health (2015). Health Profile 2015 in Indonesia from Household Health Survey (SKRT). www.depkes.go.id.
Newman MG, Takei HH, Carranza FA (2002). Clinical Periodontology, 9th Ed. Philadelphia: WB Saunders Co. p83, Pp.96-100.
Samaranayake L (2001). Essential Microbiology for Dentistry. 3rd Ed. Hongkong: Elsevier Hongkong. P 115, P 117, P 255.
Saini R (2015). A Prospective Experimental Comparative Study on the Clinical Effects of Calculus Dissolution based Oral Rinse in Gingivitis Patients. Int. J. Exp. Dent. Sci. 4(1):33-39.
Poetri ON, Soejoedono RD (2006). IgY anti Streptococcus mutans producing for anti caries. J. Agric. Indonesia 11(3):6-10.
Rajan S, Karthika S, Michael A, Gandhimathi (2011). Generation of Eggyolk Antibodies in Chicken (IgY) against *Streptococcus mutans* and its in-vitro neutralization efficacy. Arch. Appl. Sci. Res. 3(5):404-412.

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