African Journal of Microbiology Research

Volume 10 Number 40 28 October, 2016
ISSN 1996-0808

Academic Journals

ABOUT AJMR

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

The African Journal of Microbiology Research (AJMR) 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

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 40 28 October, 2016

ARTICLES

academicJournals

Vol. 10(40), pp. 1675-1684, 28 October, 2016
DOI: 10.5897/AJMR2016.8039
Article Number: 919923561328
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Anti-biofilm activity of ibuprofen and diclofenac against some biofilm producing *Escherichia coli* and *Klebsiella pneumoniae* uropathogens

Rosa Baldiris[1,4]*, Victor Teherán[1,2], Ricardo Vivas-Reyes[3,4], Alfredo Montes[1] and Octavio Arzuza[1]

[1]Grupo de Microbiología Clínica y Ambiental. Universidad de Cartagena. Facultad de Ciencias Exactas y Naturales. Programa de Biología. Campus San Pablo. Cartagena, Colombia.
[2]Farmacology Master Student. Universidad de Cartagena. Facultad de Medicina. Campus Zaragocilla. Cartagena, Colombia.
[3]Grupo de Química Cuántica y Teórica, Universidad de Cartagena, Facultad de Ciencias Exactas y Naturales, Campus San Pablo, Cartagena, Colombia.
[4]Grupo de investigación CIPTEC. Facultad de Ingeniería. Programa de Ingeniería de Procesos. Fundación Universitaria Tecnológico Comfenalco. Cartagena-Colombia.

Received 1 April 2016, Accepted 20 September, 2016.

Ibuprofen (IBU) and Diclofenac (DIC) are non-steroidal anti-inflammatory drug which have shown to have broad antimicrobial activity and limit biofilm formation. Aim of this study was to evaluate the effects of IBU and DIC against biofilm formation strong strains *Escherichia coli* and *Klebsiella pneumoniae* associated with urinary tract infections (UTIs). Additionally some virulence factors, antibiotic resistance, ESBL production were estimated to find correlation with the biofilm production and acil homoserine lactone (AHL). Our results suggest that IBU and DIC, could be useful in the treatment of urinary tract infections caused by *E. coli* and *K. pneumoniae.* IBU with CMIB 8, 30 and 125 mg/L, caused dramatic reductions in some cases and inhibited virulence factors which changes in the morphotypes expressed on congo red agar. DIC with CMIB 30 and 50 mg/L caused reductions in biofilm formation 50.1%. In this study, were evidenced the relationship between virulence factors, production of AHL and multi drugs resistance with biofilm production.

Key words: Diclofenac, ibuprofen, biofilm, acil homoserine lactone (AHL), *Escherichia coli*, *Klebsiella pneumoniae*.

INTRODUCTION

Escherichia coli and *Klebsiella pneumoniae* are the two predominant pathogens commonly isolated in urine.

*Corresponding author. E-mail: rbaldirisa@unicartagena.edu.co.

These uropathogens have also developed resistance to commonly prescribed antimicrobial agents, exerts their antimicrobial resistance against beta-lactams by producing extended spectrum beta-lactamases (ESBLs) enzymes that confers bacterial resistance to all beta-lactams except carbapenems, cephamycins and clavulanic acid (Ullah et al., 2009), severely limits the treatment options of an effective therapy and pose treatment problem resulting in high morbidity, high mortality, and increased health care costs (Schwaber and Carmeli, 2007). Uropathogenic bacteria may be responsible for many recurrent UTIs by biofilm producing. A biofilm is a complex aggregate of microorganisms in which cells adhere to each other and to a surface in a self-produced matrix of extracellular polymeric substance/slime. Biofilm production is a mechanism exhibited by several microbes to survive in unfavorable conditions. Bacteria communicate with one another using chemical signal molecules, this process of chemical communication called quorum sensing (QS), exerts a great influence on the production of virulence factors through control of transcriptional regulators as a result of changes in population density (Waters and Bassler, 2005). QS operate through a wide range of signals such as: (1) Oligopeptides, (2) Acylhomoserine lactones (AHLs), (3) Furanosyl borate (autoinducer-2), and (4) Fatty acids (McDougald et al., 2007). Most QS systems described to date in gram-negative bacteria depend on N-acyl-homoserine lactones (AHL) as signal molecules, and these QS systems found in more than 100 bacterial species (Viswanath et al., 2015).

Bacteria within biofilms are intrinsically more resistant to antimicrobial agents than planktonic cells. Antimicrobial concentrations sufficient to inactivate planktonic organisms are generally inadequate to inactivate biofilm organisms. Antibiotic resistance can increase 1000 fold (Stewart and Costerton, 2001). According to a research, more than 60% of all infections are caused by bacteria growing in biofilms (Lewis, 2001). These infections tend to be chronic as they resist innate and adaptive immune defense mechanisms, and the treatment presents a considerable unmet clinical need. To date, there are no drugs that specifically target bacteria in biofilms; however, several approaches are in early-stage (Marvig et al., 2012).

Several reports sugared when designing and testing new drug candidates, it is important that the biofilm phenotype is taken into consideration. Recently non-steroidal anti-inflammatory drug (NSAID) that specifically blocks the biosynthesis of mammalian prostaglandins by inhibiting one or both of the COX isoenzymes has been shown that dramatically decrease biofilm production in fungal and bacteria (Alem et al., 2004). In this study, we evaluated the effects of ibuprofen and diclofenac drugs against isolates from of urinary tract infection with ability to strong biofilm formation. Additionally, some virulence factors, antibiotic resistance, ESBL production, detection and identification of signaling molecules involved in the quorum sensing were estimated. This is the first this type of study conducted in Colombia.

MATERIALS AND METHODS

Bacterial strains

A total of 100 isolates obtained from patients with UTI's who were referred to two hospitals in Cartagena, Colombia, were included in this study. Specimens collected were clean catch midstream urine (54%) and urine from vesicular sounds (46%) using standard sterile procedures. The isolates were identified based on observing colonial morphology on EMB medium. Lactose-fermenting colonies were confirmed using standard biochemical tests (Voges-Proskauer, methyl red, and nitrate reduction, motility at 37°C, indole production and gas production from glucose). All the strains were stored in thioglycolate broth with 15% glycerol at -80°C. *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *Staphylococcus aureus* ATCC 25923 were used as control in all tests.

Antibiotic susceptibility testing

Antibiotic susceptibilities were determined according to the manufacturer's recommendations by overnight microdilution method with commercial dehydrated panels (NUC 60) provided by Dade Behring MicroScan (Sacramento, Calif.) that were read by the autoSCAN-4 and interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). Multidrug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012).

Detection of ESBL

Phenotypic screening of ESBL

Isolates were screened for resistance to three oxyimino-cephalosporins: Ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and the monobactam: Aztreonam (30 µg) by disk diffusion test. Zone diameters were read using criteria (CLSI, 2012). An inhibition zone of ≤17 mm ceftazidime, ≤ 22 mm cefotaxime, ≤ 19 mm ceftriazone and ≤17 mm aztreonam indicated a probable ESBL producing strain requiring phenotypic confirmatory testing.

Phenotypic confirmatory method of ESBL

ESBL production was detected by the double disc synergy test (DDST) using clavulanic acid-amoxicillin (20/10 µg) and ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg) and cefepime (30 µg) on Mueller Hinton agar as recommended by French Society for Microbiology (FSM, 2009) (http://www.sfm-microbiologie.org). The presence of ESBL was manifested by the synergistic effect of the inhibitor and discs (effect of egg, fish tail or American soccer ball) (Lezameta et al., 2010).

Analysis of biofilm formation capacity

Congo red agar method by

Suspension of tested strains was inoculated onto a specially

prepared solid medium brain heart infusión broth (BHI) supplemented with 5% sucrose and Congo red The medium was compose of BHI (37 g/L), sucrose (50 g/L), agar no.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 min, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 h at 37°C (Freeman et al., 1989).

Screening of morphotypes (Congo red agar assay)

The morphotypes of each strain was determined by the morphology of the colonies after incubation for 24 h at 37°C. All plates were visually examined and the morphotypes were categorized as: Red, dry and rough (rdar)-indicating expression of curli fimbriae and cellulose, brown (bdar), indicating expression of fimbriae but not cellulose, pink (pdar), indicating expression of cellulose, but not fimbriae and smooth and white (saw), indicating expression of neither cellulose nor fimbriae (Bokranz et al., 2005).

Microtiter plate assay (quantitative assays for biofilm formation)

A crystal violet staining method was employed to examine biofilm-forming abilities of the isolates (O'Toole and Kolter, 1998) with modifications. The isolates were inoculated into 1 mL LB broth and grown overnight at 37ºC with constant shaking. Overnight cultures were transferred to new culture medium (diluted by 1:100) and grown to OD 600 between 0.45 and 0.65 and for each strain assay, it was done in triplicate. Thirty microliters of bacteria in log phase growth were inoculated into 96-well polystyrene plates containing 100 mL fresh LB broth and incubated at 37°C for 24 h. The plates were rinsed 3 times with deionized water and the adherent bacteria cells were stained with 0.5% crystal violet for 30 min. After being rinsed 3 times with deionized water, the crystal violet was liberated by 80% ethanol and 20% acetone following a 15 min incubation. The OD values of each well were measured at 492 nm. The tested strains were classified according to the criteria of Stepanovic et al. (2007) into non-biofilm producer (OD ≤ ODc), weak biofilm producer (OD > ODc, but ≤ 2x ODc), moderate biofilm producer (OD>2x ODc, but ≤ 4x ODc), and strong biofilm producer (OD> 4x ODc).

Detection of AHL by colorimetric method

Extraction and quantification of the acyl homoserine lactone activity was performed as described by Dietrich et al. (2010). The isolates were incubated in 5 mL Mueller Hinton broth overnight at 37°C. 1.5 mL of the suspension was centrifuged at 10,000 rpm for 15 min, the supernatant was transferred and this step was repeated twice. Subsequently liquid-liquid extraction using ethyl acetate for 10 min and the organic phase (top) was removed. Next, the samples were dried at 40°C, they transferred 40 µL to a microplate and added 50 µL of 1:1 solution of hydroxylamine 2 M: NaOH 3.5 M and 50 µL of 1:1 solution of $FeCl_3$ 10% in HCl 4 M: Ethanol 95%. Finally, the optical density at 520 nm was measured in a plate reader.

Determination of AHL functional groups

N-Acyl homoserine lactone functional groups were identified by FT-IR as described by Shikh-Bardsiri and Shakibaie (2013), in five well-characterized and strong biofilm-producers *E. coli* (3) and *K. pneumoniae* (2) strains isolated. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as controls. Briefly, the AHL extract of each sample was mixed with 100 mg potassium bromide (KBr) and subjected to Shimadzu 8400S FT-IR Spectrometer; pellets were scanned at 6 cm^{-1} resolution in the spectral range of 4000 to 500 cm^{-1}. Peaks at 1764.33, 1377.99, and 1242.90 cm^{-1} correspond to the C=O bond of the lactone ring, the N=H bond, and the C-O bond, respectively. These results support the AHL data and confirm the presence of a lactone ring in the AHL (Taghadosi et al., 2015).

Anti-biofilm activity of diclofenac (DIC) and ibuprofen (IBU) on biofilm formation

The patterns IBU and DIC were obtained of Drug Analysis Laboratory (LAM), Faculty of Pharmaceutical Sciences at the University of Cartagena. The effect of IBU and DIC in solution was assessed on the ability to form biofilms by *E. coli* strains and *K. pneumoniae* previously identified as strong biofilm forming using concentrations, 2, 8, 30 and 125 mg/L IBU and 5, 15, 30 and 50 mg/L of DIC; sub-MIC concentrations as reported (Naves et al., 2010; Reślíński et al., 2013). The biofilm producer *K. pneumoniae* ATCC 700603 strain and the non-biofilm producer *E. coli* ATCC 25922 were used as controls.

The assays were done as previously mentioned for biofilm formation, after carrying out incubation in LB broth at 37°C for 24 h, 10 µL of the suspension was transferred to polystyrene plates of 96 wells, 180 µL of LB with then he added 10 µL of solution treatment and incubated for 24 h at 37°C. Washing, staining the biofilm and subsequent reading it was performed as described previously. Untreated strains were used as negative control (O´Toole and Kolter, 1998).

Minimum inhibitory concentration (MIC) was defined as the minimum concentration of a compound that produces reductions of at least half of OD 630 nm values compared with an untreated control and minimum inhibitory biofilm concentration (MBIC) of each compound was defined as the minimum concentration that reduces biofilm formation significantly compared with an untreated control. To exclude diminutions of biofilm formation due to inhibitory effects on bacterial growth, sub-inhibitory concentrations of each compound were selected for testing (Naves et al., 2010).

Statistical analysis

All assays were done in triplicate in independent assays. The effect of compounds in biofilm production was evaluated with paired two-tailed Student's t-test. $P < 0.01$ was considered significant. Statistical Package GraphPad Prism 7 was used for data analysis. P-value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Characterization of *E. coli* and *K. pneumoniae*

100, non-repeat, clinical isolates of *E. coli* (83 isolates) and *K. pneumoniae* (17 isolates) collected over a period of 12 months, were studied. Phenotypic identification of each isolate was performed based on conventional biochemical test (Table 1).

In the past years have been reported an increased incidence of UTI due to ESBL-producing *E. coli* and *K. pneumoniae*. In this study we found that ESBL production was seen in 44 (23.81%) isolates of Enterobacteriaceae. ESBL production was higher in *E. coli* followed by *K.*

Table 1. Biochemical tests used to identify *E. coli* and *K. pneumoniae*.

Biochemical test	*E. coli*	*K. pneumoniae*
Catalase	+	+
Triple sugar agar TSI	+	+
Indole production	+	-
Methyl red	+	-
Urease	-	+
Voges proskauer	-	+
Simmon´s citrate	-	+
H_2S	-	-

*Results based on the types of substrate utilization: Positive (+) and negative (-).

Figure 1. ESBL production double disc synergy test (DDST) using clavulanic acid-amoxicillin (20/10 µg) and ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg) and cefepime (30 µg) on Mueller Hinton agar.

pneumoniae. 83% of the strains identified as *E. coli*, found that 12.05% were ESBL producers and for *K. pneumoniae* total (17%) strains were 11.76% ESBL-producing strains (Figure 1).

These findings are in agreement with previous studies such as was reported by Akram et al. (2007) and Kalsoom et al. (2012) on urinary infection also showed that *E. coli* and *Klebsiella* spp. are the most common urinary pathogens in UTI. A study by de Paz et al. (2015) reported a higher prevalence of ESBL-producing strains of *E. coli* that found in this research with 21.5%. As it has been reported that the overall prevalence of ESBL producing *K. pneumoniae* is about of 17.7% (Iabadene et al., 2008).

In this contribution we report an antimicrobial activity and outcomes in 100 isolates subject to biofilm detention. The antimicrobial resistance profiles for the isolates were determined using the MicroScan Gram Negative Urine Combo Panel Type 60 of 20 antimicrobials and breakpoints for classification as resistant used were determined according to the guidelines of the (CLSI, 2010) (Table 2).

The outcomes of susceptibility testing performed on the panel MicroScan show that only 6 of the 22 antibiotics tested (amikacin, doripenem, ertapenem, meropenem and combinations cefotaxime/acid clavulanate, ceftazidime/clavulanic acid) were effective against more than 95% of the strains tested.

Prakash et al. (2013), reported similar data to those obtained where the greatest resistance of *E. coli* and *K. pneumoniae* to antibiotics such as ciprofloxacin, ceftazidime, cefotaxime and ceftriaxone. And they are also very sensitive to antibiotics such as carbapenems meropenem and imipenem, amikacin, and aminoglycoside.

In our study, was observed that half of antibiotics evaluated showed a degree of resistance between 40 and 50% of the strains, and even antibiotics often used as ciprofloxacin and ampicillin, it was found that 55 and 75% respectively of the strains were resistant. In addition, they identified about 20% of strains with ESBLs were so resistant to antibiotics such as aztreonam, cefotaxime, ceftazidime, and ceftriaxone. 60% of the strains were sensitive to antibiotics such as ceftriaxone, aztreonam and ceftazidime. For cefotaxime and amoxicillin/ clavulanate a smaller percentage, 42 and 45% respectively was found. And more than 20% of strains were resistant to these antibiotics, highlighting cefotaxime, in which 40% of the strains were resistant and 20% intermediate. These data are confirmatory of susceptibility testing by the MicroScan panel, obtaining again, resistance in 60% of strains with the group of antibiotics evaluated compared with 55% obtained in antibiogram. These outcomes are similar to those reported previously by Arce et al. (2012) where it was found that the resistance of *E. coli* and *K. pneumoniae* isolated from patients with UTI against ceftazidime, cefotaxime, ceftriaxone, and aztreonam are about 40, 45, 32 and 20%, respectively (Stepanović et al., 2004).

The rate of multiple antibiotic resistance (MAR) was determined for each of the strains. 65% of isolates had an index ≥ 0.2, indicating that the bacterium has multiple resistance (Vanegas et al., 2009). These outcomes have a huge impact when selecting empiric treatment of these infections. Since, in general, *E. coli*, as in other studies (Alós et al., 2005; Andreu et al., 2005), shows a high rate of resistance to most of the antibiotics greater clinical use. The use of antibiotics with limited antimicrobial activity in the ITU is associated with lower clinical efficacy in pyelonephritis, with more failures and less removal, and serious infections were associated with increased mortality, and therefore must be avoided as initial empiric treatment these infections (Gomez, 2007). The choice of antibiotic treatment for UTI must be based on the results of urine culture and sensitivity testing. However, in

Table 2. Antibiotic susceptibility pattern of *E. coli* and *Klebsiella* species isolates.

Antimicrobial Agent	Interpretive break points		S (%)	R (%)	I (%)
	Susceptible	Resistant			
Amikacin	≤16	≥64	96	2	2
Amp/Sulbactam	≤8/4	≥32/16	34	46	20
Ampicilin	≤8	≥32	25	75	0
Aztreonam	≤8	≥32	72	27	1
Cefazolin	≤8	≥32	61	31	8
Cefepime	≤8	≥32	73	27	0
Cefotaxime	≤8	≥64	73	27	0
Cefoxitin	≤8	≥32	88	7	5
Ceftazidime	≤8	≥32	68	28	4
Ceftriaxone	≤8	≥64	73	24	3
Ciprofloxacin	≤1	≥4	46	50	4
Doripenem	≤0,5	≥	99	1	0
Ertapenem	≤2	≥8	99	1	0
Gentamicin	≤4	≥16	74	25	1
Meropenem	≤4	≥16	98	2	0
Nitrofurantoin	≤32	≥128	89	6	5
Pip/tazo	≤64/4	≥128/4	83	11	6
Piperacilin	≤16	≥128	52	45	3
Tobramycin	≤4	≥16	66	28	6
Trimet/sulfa	≤2/38	≥4/76	51	49	0

situations in which the symptoms or the patient's condition does not allow wait to get this information. It is important to know which antibiotic to use empirically in this initial period, it relied on the knowledge of local resistance treatment (Hawser et al., 2011).

Based on these results, it is possible to suggest that the treatment regimen of patients with UTI should be geared to the use of carbapenem drugs, as well as the combination of cefotaxime/clavulanate or ceftazidime/ clavulanate. Also, the use of amikacin aminoglycoside would have enough sense. However, should take into account the risk of ototoxicity and nephrotoxicity with the latter, as well as the location of the UTI, whether adult or child, whether pregnant or not and this way to individualize the patient. But these results mark a reference point for further *in vivo* investigations, which provide greater knowledge to make recommendations more evidence on the treatment schedule of the patients with UTI.

Detection and quantification biofilm formation

For this study, 99% of the strains analyzed presented biofilm forming activity in some degree. 1% of clinical isolates not form biofilm, 45% of the strains showed weak and biofilm formation 49% showed moderate activity. 5% of the isolates were strong ability to form biofilms; *E. coli* was 3 and K. *pneumoniae* was 2 (Figure 2).

In other studies on bacterial resistance and biofilm formation in *E. coli* and *Klebsiella* spp. you can see the same trend in the distribution of the groups. Shikh-Bardsiri and Shakibaie (2013), reported in its study for a total of 88 isolates the percentages of non-forming strains and weak, moderate and strong production capacity biofilms were 6.8, 36, 39.7 and 17.4%, respectively. Furthermore, a relationship between biofilm formation and antibiotic resistance was observed, since most strains with multiple resistance, are the same as had higher optical densities for biofilm formation, strains with biofilm-forming ability of moderate and strong. This relationship, multidrug resistance-biofilm formation has been reported by Lopez et al. (2008) and Hirales-Casillas et al. (2009), so their importance in the clinical setting for proper patient management. In this communication, we also report the ability of primary uropathogens isolates of *E. coli* and *K. pneumoniae* to express curli fimbriae and cellulose on LB agar without salt, which is coupled to the biofilm-formation capability of the organism. Three different morphotypes were found, 50% of the isolates was rdar, 27.3% bdar and 22.7% rdar.

Detection of AHL by colorimetric method

For quantification of AHL optical density it was measured in a microplate reader at a wavelength of 520 nm (Figure 3). The AHL was no or only weak activity for those

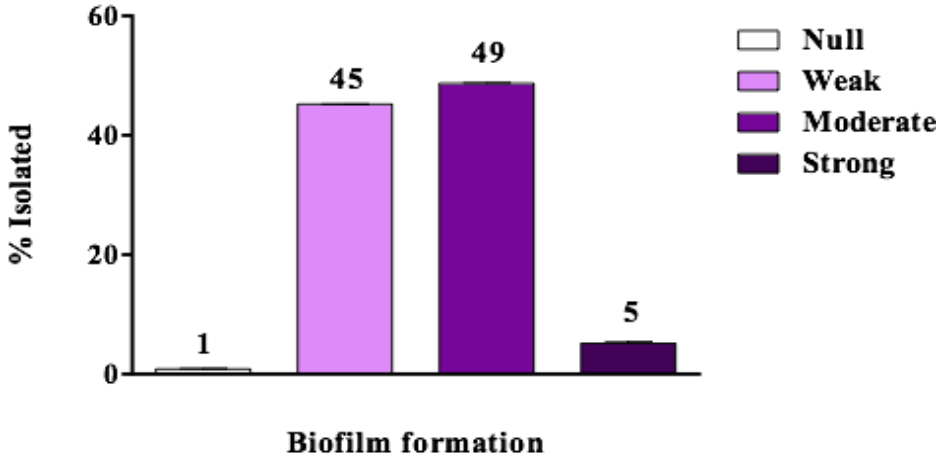

Figure 2. Classification of isolates according to their ability to form biofilms. Error bars represent the standard deviation from the mean of three observations.

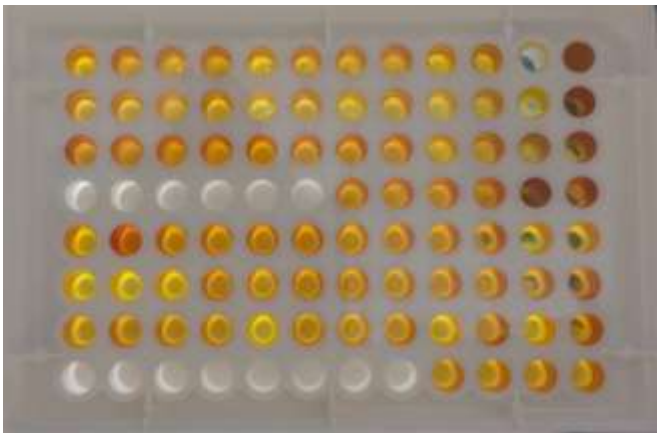

Figure 3. Quantification of AHL (optical density) measured in a microplate reader at a wavelength of 520 nm.

isolates that showed an optical density ≤0.98. Strains that showed activity AHL are those that were strong forming biofilms (5 strains), one of the strains that is within this group did not exceed the threshold of activity AHL, however, it was close to the limit with a value of 0.962 (Figure 4). Control strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603. AHL activity showed values below the threshold, 0.720 and 0.414, respectively. Only four isolates showed activity AHL above the threshold, the highest activity was 1.329 (*E. coli)*, followed by 1.190 (*E. coli*), 0.993 (*K. pneumoniae* and *E. coli*).

Several studies have reported that *E. coli* is not able to produce AHL, yet has the ability to alter their pattern of gene expression and phenotypic properties in response to AHL by the AHL responsive transcriptional regulator SdiA, according to the presence of other locations of Gram-negative bacteria (Ahmer, 2004). Although the association between biofilm formation and other virulence factors has been variable, in the present study biofilm formation in *E. coli* and *K. pneumoniae* strains capable of producing relatively high AHL was detected. Already has been reported in recent studies by Taghadosi et al. (2015), uropathogenic *E. coli* strains producing biofilms with capacity to produce and communicate through AHL molecules type. More research is needed to understand the molecular genetics of this compound in *E. coli*.

Determination of AHL functional groups

Strains with the highest activities were selected for AHL analysis of functional groups by FT-IR spectroscopy (Figure 5). The results show amide carbonyl bond absorptions I and II was found at 1547 and 1516 cm^{-1} and characteristic absorptions near 1678 and 1647 cm^{-1} these signals are attributable to the presence of γ-lactone and ketone carbonyls. The band bending vibration of the O=CN bond was found to 680 cm^{-1} (Wang et al., 2011; Yang et al., 2006). These outcomes support the activity data AHL and confirm the presence of a lactone ring in producing strains biofilm with high activity AHL; however the spectra obtained for the extracts have bands too broad and in some cases overlap with other bands, due to the presence of other compounds in the extracts. So this technique is not allowed to obtain clear results apparently by low concentrations to those found in the AHL extract (Cuadrado Silva, 2009).

Effect of soluble compounds on bacterial growth and biofilm formation

IBU reduced biofilm formation significantly in three of the five strains, of which two are *K. pneumoniae* and is *E.*

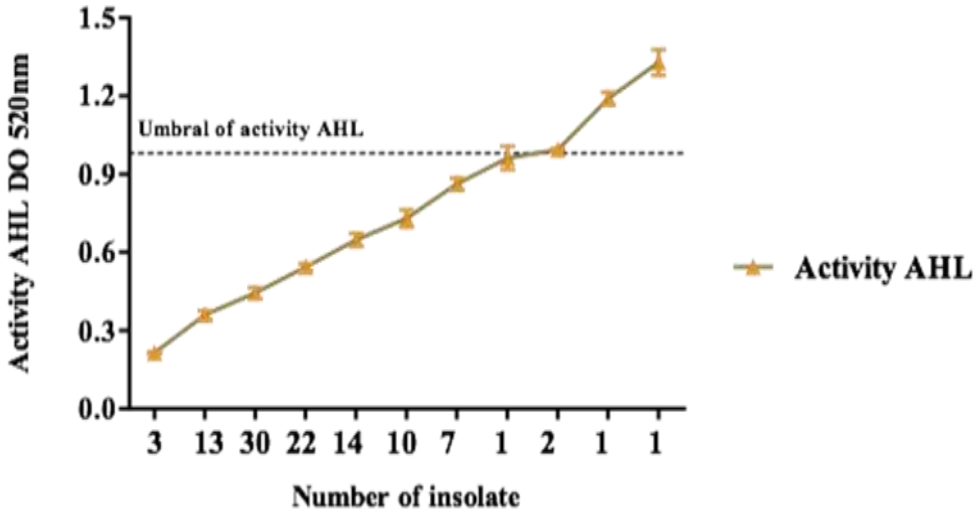

Figure 4. AHL- activity by uropathogenic *E. coli* and *K. pneumoniae* exhibiting strong biofilm. The results below threshold indicate weak or no AHL activity.

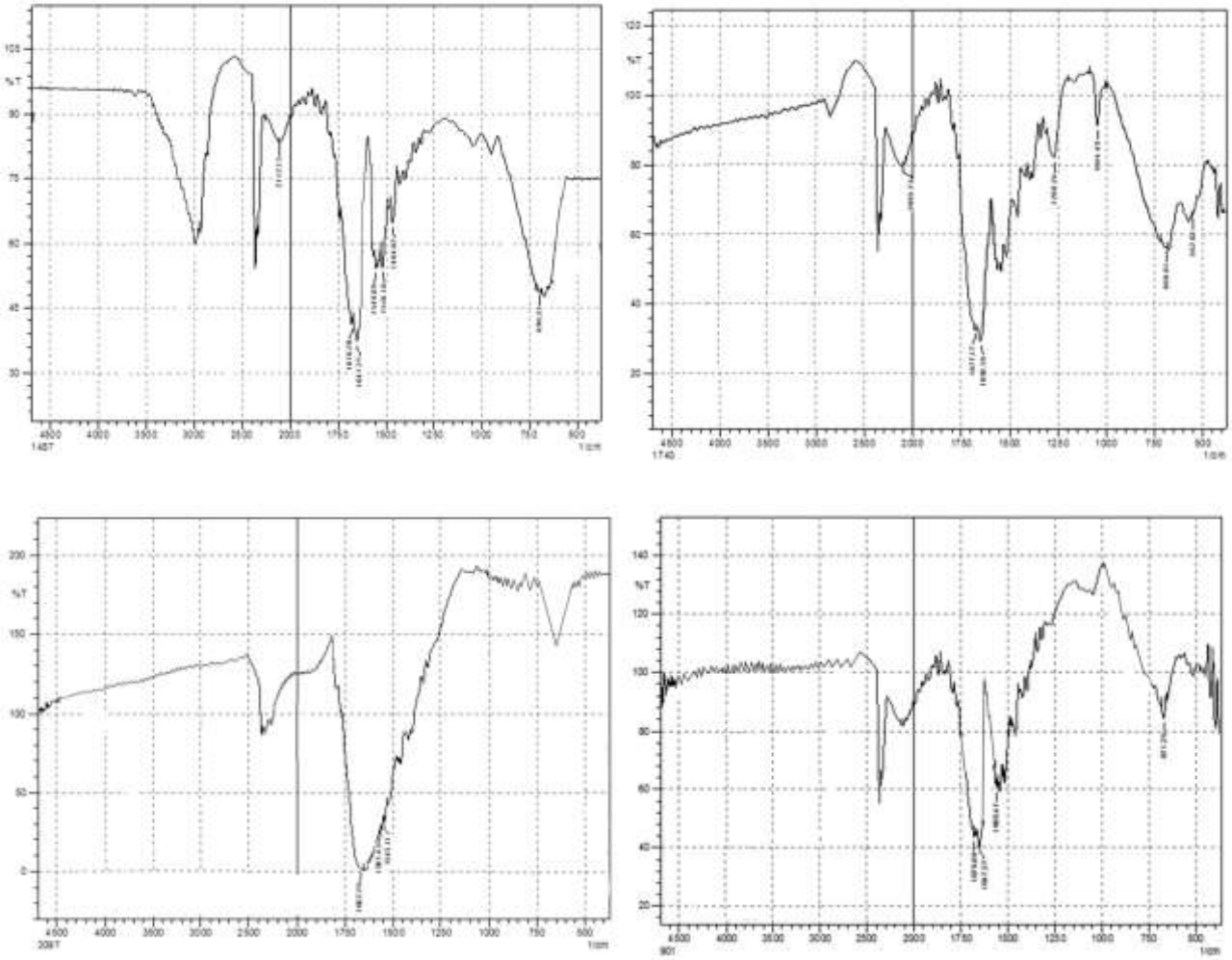

Figure 5. FT-IR spectra of AHL functional groups produced by uropathogenic *E. coli.* The AHL was extract from organism by LLE- methods as described in the text. The pure compound was then subjected to FT-IR spectroscopy. The lactone ring was shown at 1764.69 cm^{-1} wave number.

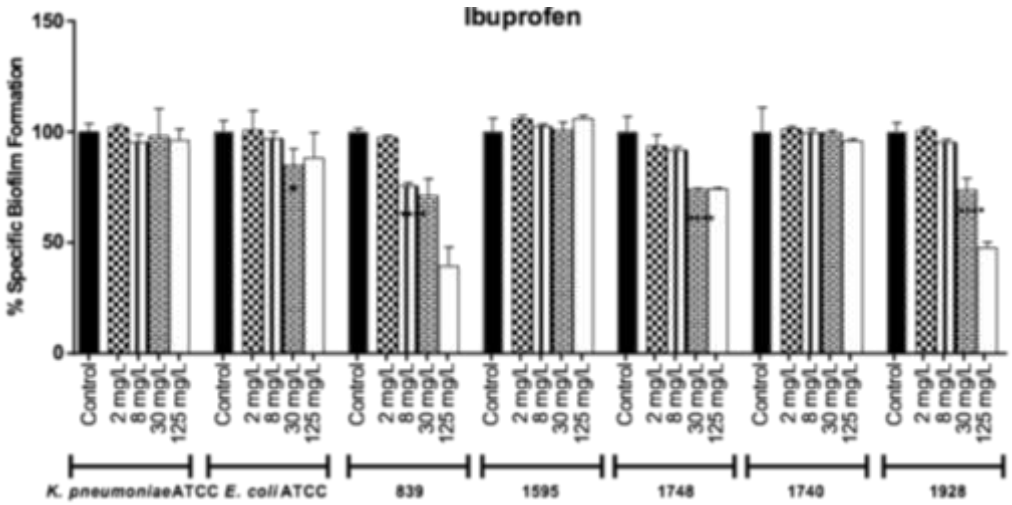

Figure 6. Ibuprofen effect on biofilm formation in *E. coli* strains *K. pneumoniae* strong forming and biofilm.

(A) **(B)**

Figure 7. Morphotypes on red Congo agar. A. rdar that displayed cellulose and curli fimbriae. B. bdar that displayed only curli fimbriae, but not cellulose.

coli, with CMIB 8 and 30 mg/L, decreases observed 20 to 25%. While in some cases the presence of the highest concentration (125 mg/L) caused dramatic reductions in biofilm formation, ranging from 52.4 to 60.6% (Figure 6). Naves et al. (2010), studied the effects of ibuprofen on biofilm formation in *E. coli* strains, identified as forming strong biofilm. In this study it was found that the concentration of IBU in urine obtained under a treatment regimen is much greater than the concentration of IBU to reduce biofilm formation by five of the seven strains of *E. coli* tested. Therefore, IBU administered at standard doses may prevent biofilm formation by *E. coli* in indwelling urinary catheters. These outcomes can be as was explained by Naves (2010) and coworkers when they detected not biofilm reduction were observed, suggesting that IBU could be washed out from the wells during the treatment. With the consequence that the concentration of IBU is down more than usual.

A study performed by Drago et al. (2002), reports that sub-MIC concentrations IBU inhibit the production of fimbriae by *E. coli* strains, to reduce bacterial adhesion uro-epithelial cells. However, in our study we can demonstrate that the sub-MIC concentrations at which biofilm formation is inhibited, the morphotype expressed by these strains incubated with sub-MIC concentrations (8 and 30 mg/L) on Congo red agar changes and evidence inhibiting production of fimbriae (Figure 7).

Moreover, bacterial hydrophobicity and hemolysin production by *E. coli* strains are also affected by incubation with drug (Drago et al., 2002). Also, it has

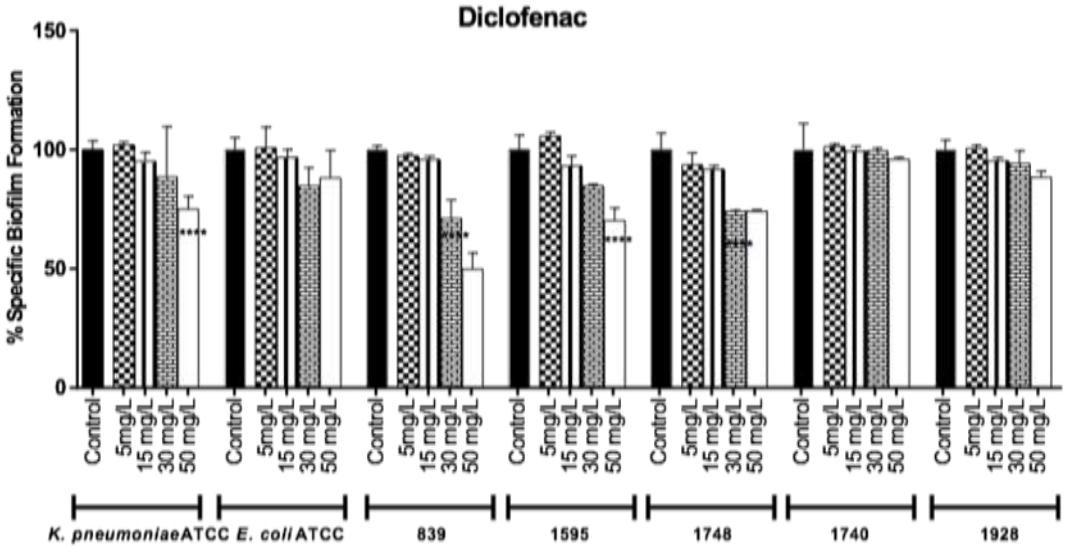

Figure 8. Diclofenac effect on biofilm formation in *E. coli* strains *K. pneumoniae* strong forming and biofilm.

demonstrated anti-adhesive effect of IBU in *Candida albicans* using a disc model system *in vitro* catheter (Alem and Douglas, 2004). Additionally, some *in vivo* studies have reported beneficial effects when combined IBU and antibiotics for the treatment of otitis media and experimental pneumococcal in a rat model of chronic pulmonary infection with *P. aeruginosa* (Diven et al., 1995).

DIC reduced biofilm formation significantly in three of the five isolates, of which two are *K. pneumoniae* and the other is *E. coli*, with CMIB 30 and 50 mg/L respectively, decreases observed 26 to 30%. Even for a sample found that the presence of the highest concentration (50 mg/L) caused reductions in biofilm formation 50.1% (Figure 8). Has been reported that diclofenac and ibuprofen at concentrations equal to those obtained from human blood drugs (serum) inhibits biofilm formation of *S. aureus* and *E. coli* in a polypropylene mesh surface (Reśliński et al., 2013). A strain of *E. coli* (1740), showed not significant changes in biofilm formation with exposure to IBU and DIC. And all strains evaluated when treated with these compounds showed not significant increase in biofilm forming capacity relative to the control. So far there has evaluated the effect of IBU and DIC in biofilm formation in *K. pneumoniae* strains. However other studies have reported compounds such as alkaloids, chitosan, linoleic acid, curcumin and eugenol (Magesh et al., 2013) and N-acetyl cysteine, which has been used at a concentration of 2000 mg/L inhibits growth of a variety of Gram-positive and negative organisms, including *K. pneumoniae*, *E. cloacae* and *E. coli* (Olofsson et al., 2003).

In tis contribution was found that isolates with the highest AHL activities also exhibited strong adherence to microplate wells (P≤0.05). A significant relationship between strong biofilm production and production of AHL, suggesting the importance of this mechanism in bacterial pathogenicity and its role in sensitivity to antibiotics. IBU and DIC reduced biofilm formation significantly in three of the five strains, *K. pneumoniae* and *E. coli*, IBU with CMIB. While in some cases the presence of the highest concentration (125 mg/L) caused dramatic reductions in biofilm formation, ranging from 52.4 to 60.6%. Our results suggest that IBU and DIC could be useful in the treatment of urinary tract infections caused by *E. coli* and *K. pneumoniae* due to their inhibitory effect on both bacterial growth and biofilm formation.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

R.B is indebted to the Universidad de Cartagena (Cartagena, Colombia) for continuous support to her group. A.M thank the Administrative Department of Science, Technology and Innovation Colciencias for its 'Young researchers' program. The authors would like to thank Dr. Maricela Viola and Dr. Julia Pimienta for their important technical and clinical support.

REFERENCES

Ahmer BM (2004). Cell-to-cell signalling in *Escherichia coli* and *Salmonella* enterica. Mol. Microbiol. 52(4):933-945.

Akram M, Shahid M, Khan AU (2007). Etiology and antibiotic resistance

patterns of community-acquired urinary tract infections in JNMC Hospital Aligarh, India. Ann. Clin. Microbiol. 6(1):1-4.
Alem MA, Douglas LJ (2004). Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of Candida albicans. Ann. Agents Chemother. 48(1):41-47.
Alós J, Serrano MG, Gómez JL, Perianes J (2005). Antibiotic resistance of *Escherichia coli* from community-acquired urinary tract infections in relation to demographic and clinical data. Clin. Microbiol. Infect. 11(3):199-203.
Andreu A, Alós J, Gobernado M, Marco F, de la Rosa M, García J, Grupo CEPESAPU (2005). Etiología y sensibilidad a los antimicrobianos de los uropatógenos causantes de la infección urinaria baja adquirida en la comunidad. Estudio nacional multicéntrico. Clin. Microbiol. Enferm. Infect. 23(1):4-9.
Arce Z, Alarcón E, Limo J, Llontop J, Valle J (2012). Detección de genes shv y tem en cepas de *Escherichia coli* productoras de B-lactamasas de espectro extendido procedentes de dos centros hospitalarios de Chiclayo-Perú: enero-agosto 2011. R. Med. Nac Almanzor. 5(3):13-16.
Bokranz W, Wang X, Tschape H, Romling U (2005). Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J. Med. Microbiol. 54:1171-1182.
Clinical and Laboratory Standards Institute, CLSI (2010). Performance Standards for Antimicrobial Susceptibility Testing. Twentieth Informational Supplement M100- 20.
Clinical and Laboratory Standards Institute, CLSI (2012). Performance standards for antimicrobial susceptibility testing. M100-21.
de Paz RA (2015). *Klebsiella pneumoniae* and *Escherichia coli* as betalactamasas producers in patients with urinary tract infection. *R.* Cub. Med. Int. 14(4):16-29.
Dietrich J, Mckee A, Keasling J (2010). High-Throughput Metabolic Engineering: Advances in Small-Molecule Screening and Selection. Rev. Bioche 79:563-590.
Diven F, Evans W, Alper C, Burckart J, Jaffe R, Doyle J (1995). Treatment of experimental acute otitis media with ibuprofen and ampicillin. Int. J. Pediatr. Otorhinolaryngol. 33(2):127-139.
Drago L, De Vecchi E, Nicola L, Valli M, Gismondo MR (2013). Effects of subinhibitory concentrations of ibuprofen isobuthanolammonium on virulence factors of uropathogenic *Escherichia coli*. J. Chemother. 14(3):314-5.
Freeman DJ, Falkiner FR, Keane CT (1989). New method for detecting slime production by coagulase negative staphylococci. J. Clin. Pathol. 42:872-874.
Gómez JG (2007). Infección urinaria por *Escherichia coli* multirresistente: impacto clínico y nuevas perspectivas. Med. Clín. 129(11):412-413.
Hawser SP, Bouchillon SK, Lascols C, Hackel M, Hoban DJ, Badal RE, Livermore DM (2011). Susceptibility of *Klebsiella pneumoniae* isolates from intra-abdominal infections, and molecular characterization of ertapenem-resistant isolates. Antimicro. Ang. Chemother. AAC-00070.
Hirales CE (2009). Bacterias multirresistentes formadoras de biopeliculas en el área de terapia intensiva de un hospital de la ciudad de chihuahua. Cong. Nac. Biotecnol. CVI-69.
Iabadene H, MessaiY, Ammari H, Ramdani-Bouguessa N, Lounes S, Bakour R, Arlet G (2008). Dissemination of ESBL and Qnr determinants in Enterobacter cloacae in Algeria. Antimicrob. J. Chemother. 62(1):133-136.
Kalsoom BANO, Jafar KHAN, Begum H, Munir S, Ulakbar N, Ansari JA, Anees M (2012). Patterns of antibiotic sensitivity of bacterial pathogens among urinary tract infections (UTI) patients in a Pakistani population. Afri. J. Microbiol. 6(2):414-420.
Lewis K (2001). Riddle of biofilm resistance. Antimicrob. Agents Chemother. 45(4):999-1007.
Lezameta L, Gonzáles E, Tamariz J (2010). Comparación de cuatro métodos fenotípicos para la detección de beta-lactamasas de espectro extendido. Rev. Peru. Med. Exp. 27(3):345-351.
López J, Moorillón G (2008). Biopelículas en la resistencia de bacterias aisladas. Synthesis. Octubre-Noviembre.
Magesh H, Kumar A, Alam A, Priyam P, Sekar U, Sumantran N, Vaidyanathan R (2013). Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae.*
Magiorakos A, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C,Marvig R L, Søndergaard MS, Damkiær S, Høiby N, Johansen HK, Molin S, Jelsbak L (2012). Mutations in 23S rRNA confer resistance against azithromycin in Pseudomonas aeruginosa. A. Agents Chemother. 56(8):4519-4521.
McDougald D, Rice SA, Kjelleberg S (2007). Bacterial quorum sensing and interference by naturally occurring biomimics. Anal Bioanal. Chem. 387:445-453.
Naves P, Del Prado G, Huelves L, Rodriguez V, Ruiz V, Ponte M, Soriano F (2010). Effects of human serum albumin, ibuprofen and N-acetyl-L-cysteine against biofilm formation by pathogenic *Escherichia coli* strains. Hosp. J. Infect. 76(2):165-170.
Olofsson C, Hermansson M, Elwing H (2003). N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. Appl. Microb. Environ. 69(8):4814-4822.
O'Toole G, Kolter R (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28(3):449-461.
Paterson D (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18(3):268-281.
Prakash D, Saxena RS (2013). Distribution and antimicrobial susceptibility pattern of bacterial pathogens causing urinary tract infection in urban community of Meerut City, India. ISRN microbiol.
Reśliński A, Dąbrowiecki S, Głowacka K (2013). The impact of diclofenac and ibuprofen on biofilm formation on the surface of polypropylene mesh. Hernia. 19(2):179-185.
Schwaber MJ, Carmeli Y (2007). Mortality and delay in effective therapy associated with extended-spectrum beta-lactamase production in Enterobacteriaceae bacteraemia: a systematic review and meta-analysis. J. Antimicrob. Chemother. 60(5):913-20.
Shikh H, Shakibaie MR (2013). Proteus Strains Isolated from Hospitalized Patients; Matter of Hospital Hygiene and Antimicrobial Stewardship. Pak. J. Biol. Sci. 16(22):1496-1502.
Silva C, (2009). Aislamiento de n-acilhomoserinlactonas de algunas bacterias procedentes del mar Caribe colombiano, como evidencia de la existencia de circuitos de quorum sensing. Doctoral dissertation. U. Nac. Col.
Stepanovic S, Ćirković I, RaninL (2004). Biofilm formation by *Salmonella* spp. and Listeria monocytogenes on plastic surface. Lett. Appl. Microbiol. 38(5):428-432.
Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I, Ruzicka F (2007). Quantification of biofilm in microtiter plates:overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115:891-899.
Stewart PS, Costerton JW (2001). Antibiotic resistance of bacteria in biofilms. Lancet 358:135-138.
Taghadosi R, Shakibaie MR, Masoumi S (2015). Biochemical detection of N-Acyl homoserine lactone from biofilm-forming uropathogenic *Escherichia coli* isolated from urinary tract infection samples. Rep. Bioche. Mol. Biol. 3(2):1-6.
Ullah F, Malik SA, Ahmed J (2009).Antibiotic susceptibility pattern and ESBL prevalence in nosocomial *Escherichia coli* from urinary tract infections in Pakistan. Afr. J. Biotechnol. 8(16):3921-3926.
Vanegas M, Correa N, Morales A, Martínez A, Rúgeles L, Jiménez F (2009). Resistencia a antibióticos de bacterias aisladas de biopelículas en una planta de alimentos. Rev. MVZ Córdoba 14(2).
Wang J, Quan C, Wang X, Zhao P, Fan S (2011). Extraction, purification and identification of bacterial signal molecules based on N-acyl homoserine lactones. Microb. Biotechnol. 4(4):479-490.
Waters CM, Bassler BL (2005). Quorum sensing: Cell-to-cell communication in bacteria. Ann. Rev Cell. Dev. Biol. 21:319-46.
Yang Y, Lee T, Kim J, Kim E, Joo S, Lee S, Kim G (2006). High-throughput detection method of quorum-sensing molecules by colorimetry and its applications. Anal. Biochem. 356(2):297-299.

academicJournals

Vol. 10(40), pp. 1685-1693, 28 October, 2016
DOI: 10.5897/AJMR2016.8194
Article Number: 860A4E461330
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Pneumonia in Algerian Ouled Djellal sheep: Bacteriological study and macroscopic aspect of lung lesions

Kamel Akloul* and Mohammed Nabil Menoueri

Veterinary Institute, University of Blida, Algeria.

Received 4 July 2016, Accepted 15 September, 2016.

Respiratory diseases in sheep are a multifactorial etiology syndrome, causing great economic losses. This study was carried out with the aim of establishing a bacteriological diagnosis of respiratory diseases in sheep, on the basis of lungs with macroscopic lesions taken from sheep slaughtered at the Blida abattoir. A total of 150 samples (swabs and lung parenchyma) from 75 Ouled Djellal sheep, aged six to twelve months old, with pulmonary lesions, were collected to determine possible correlations between the etiological agents and the type of lesion. Hepatization (or consolidation) was the main lesion observed (70%), preferentially localized on the right apical lobe (88% of the cases). This is a special form of pneumonia called atypical pneumonia. A varied microbial flora was isolated, alone or in association, namely bacteria with respiratory tropism as well as others of secondary infection. The bacteria most frequently isolated were γ-hemolytic streptococci (18%), *Escherichia coli* (17.5%), *Micrococcus* species (14.5%), and coagulase-negative staphylococci (10.5%); the large family of Enterobacteriaceae represented nearly 43% of the isolates. The pneumotropic bacteria (*Mannheimia haemolytica* and *Pasteurella multocida*) which count for 5.6% of the isolates, turned out to be correlated with the hepatization lesions.

Key words: *Mannheimia haemolytica*, *Pasteurella multocida*, pneumonia, sheep, Algeria.

INTRODUCTION

Sheep farming in Algeria constitutes 50% of the agricultural gross domestic product. The sheep number has increased from 17.5 to 26.6 million head with an average annual increase of 4.4% over the ten-year period (2003 to 2013) (MADR, 2014). In 2003, it represented 25 to 30% of animal production and 10 to 15% of agricultural production. It provides more than 50% of the total meat needs of the country (Abdelguerfi and Ramdane, 2003). The rearing of grazing livestock in Mediterranean countries includes 13% of the world's sheep and goats (Boutonnet, 1998). Respiratory diseases in sheep are among the most frequent pathologies, with heavy health and economic impacts. They form a multi-factorial complex involving the interaction of host, environment,

*Corresponding author. E-mail: akl_kamel@yahoo.fr. Tel: +213774856703.

as well as a wide variety of pathogens. The term respiratory disease complex (RDC) is used for the condition conventionally known as bronchopneumonia. RDC is a polymicrobial infection that develops when the immune system of the animal is compromised by stress factors such as crowding, transportation, draught and inclement weather combined with increased exposure to pathogens, and may lead to respiratory infection (Asaye et al., 2015).

Environmental conditions and poor hygiene prevailing in Algerian farms are all stress causing factors that promote the onset of respiratory diseases. When they do not involve the death of the subject, they result in debilitating medical conditions and therefore a decreased carcass value. Indeed, respiratory troubles in lambs cause mortality, growth reduction and economic losses, due to drug costs and condemnations in abattoirs (Goodwin et al., 2004). The role of stress in the natural incidence of the pulmonary form of pasteurellosis is clearly highlighted by the fact that the disease onset is associated with a sudden source of stress such as exposure to an extreme temperature (hot or cold) with a high level of humidity, overcrowding, poor ventilation, bad raising practices, rough handling, transport over long distances, excessive dust, high load of internal or external parasites and the mixing of animals from different sources (Sharma et al., 1990; Martin, 1996).

Small ruminants are especially sensitive to respiratory infections, mostly as a result of deficient management practices that make these animals more susceptible to infectious agents. The tendency of these animals to huddle and group rearing practices further predispose small ruminants to infectious and contagious diseases (Chakraborty et al., 2014). In a study in Ireland, McIlroy et al. (1989) demonstrated a significant correlation between the percentage of seized sheep carcasses due to pleurisy or pneumonia and rainfall, wind, temperature as well as humidity. Galapero et al. (2016) used Bayesian networks to identify risk factors for pulmonary consolidation. The results showed that the main factors causing ovine inflammatory respiratory processes and pulmonary consolidation were temperature, relative humidity, and *Mycoplasma* species. Control of these factors may help reduce the incidence of pulmonary consolidation. In sheep, the pathogenesis of respiratory diseases is difficult to determine because of the interaction of different causative agents which may have similar pathological models (Alley, 1975). Of all the organ systems, the respiratory tract may be unique in its vulnerability to injurious agents. Nowhere else in the body, such a vast surface area (approximately 100 m^2) is directly exposed to airborne pathogens at about 20 times/min. Not only is the area and exposure extreme, but the underlying blood circulation is only two cell layers of about 0.5 nm each, removed from the alveolar surface (Asok et al., 2014).

Previous or combined infection with some respiratory viruses may increase the susceptibility of farm animals to secondary bacterial pneumonia (Cutlip et al., 1993; Ozyildiz et al., 2013). Sheep are particularly sensitive to parainfluenza virus type 3 (PIV-3) and bovine respiratory syncytial virus (BRSV); antibodies against these viruses have been demonstrated in this species (Lemhkuhl et al., 1985; Giangaspero et al., 1997). An estimated 40% of viral respiratory infections are complicated by bacterial pneumonia (Loosli, 1968). *Pasteurella,* which is the main bacteria responsible for lung disease in sheep, acts as secondary infection agents after a viral or mycoplasma infection (Douart, 2002). Whatever the initial cause of lung damages is (environmental, viral, bacterial or parasitic), *Mannheimia haemolytica* is often found as a complicating agent. Ovine pneumonia due to *M. haemolytica* is the major cause of perinatal mortality (10 to 40%) depending on the farm type and the season (Malone, 1988). In outbreaks of acute ovine and caprine pneumonia, *Pasteurella* especially *M. (Pasteurella) haemolytica* and *Pasteurella multocida* have been isolated more than other pathogenic agents from affected lungs. About 30% of domestic animal mortality is known to be related to *Pasteurella* infection (Valadan et al., 2014).

Pasteurellosis, also called "enzootic pneumonia", does not correspond to a specific nosologic entity, but *Pasteurella*, which are the most frequently isolated pathogens, are therefore considered as the main cause of the disease (Zrelli, 1988). Atypical pneumonia is defined to distinguish a rather similar form of pasteurellosis, but without the fatal nature of this latter (Rouchy, 1992), combined with a multitude of pathogenic agents including *Mycoplasma ovipneumoniae*. Due to the high losses resulting from these pathologies and considering the importance of the national herd (over 26 million heads), it was deemed appropriate to carry out an investigation to determine a first estimate of the prevalence of pneumonia in sheep, by studying pulmonary lesions observed in the slaughter house to try and establish a link between macroscopic lung lesions and bacterial agents, especially *Pasteurella* species.

MATERIALS AND METHODS

The study focused on sample "all comers" of Ouled Djellal sheep slaughtered at Blida abattoir (Algeria) from January 2009 to April 2010. These animals came from different farms and neighboring regions of Blida (15 km from a large cattle market), where farming conditions are substantially the same. It concerned only males, aged 6 to 12 months.

Blida is located at 36°28'North and 2°49' East, at an altitude of 272 m above sea level. It extends over 1478.62 km^2. The temperatures in the area vary from 2°C in winter (January) to 45°C in summer (July) and the average annual precipitation is around 600 mm per year, reaching its peak from December to February (30 to 40% of annual precipitation).

According to the local agriculture service, the number of sheep in the study area is about 40,000 heads. Blida abattoir was selected because it is the largest in the region in terms of slaughter

Table 1. Frequency distribution of gross pathological lesions in lungs of sheep observed at Blida slaughterhouse.

Lesion	Number	%
Red hepatization	39	52.0
Grey hepatization	14	18.7
Atelectasis	10	13.3
Emphysema	8	10.7
Others*	4	05.3
Total	75	100.0

*Congestion, Abscesses.

capacities (around 500 head of sheep/day).

Macroscopic study of lung lesions

A survey sheet was established for each animal with lungs lesions. The lesions were described according to its precise location, degree of expansion, appearance (color), consistency, volume, shape and the nature of the macroscopic pathological changes.

Lesion score

For each lobe, the extent of the lung lesion was visually evaluated and measured using a 4-point scale, from score 0 (no injury), score 1 (≤ 25% of the lobe), score 2 (25 to 50%), score 3 (50 to 75%) to score 4 (> 75% of the lobe). The overall score was obtained by summing the scores for each lobe between 0 and 32.

Sample collection

A total of 75 bronchial swabs and 75 lung parenchyma were collected for bacteriological analysis. Two samples types were taken from freshly slaughtered animals with gross lesions suggesting pneumonia: a swab of the bronchi using a sterile swab after sectioning of the injured tissue, and sectioning a fragment of pulmonary fabric from the same compromised parenchyma.

These samples were placed in insulated boxes at +4°C, to be sent to the laboratory as soon as possible. For practical reasons, the samples were frozen (-20°C) in order to be exploited subsequently. Each sample, properly identified and accompanied by an identification card was placed in airtight individual packaging, which no added preservative or antiseptic.

Bacteriological analysis

The bacterial analysis carried out at the Microbiology Laboratory of the Veterinary Institute (University of Blida) focused on the isolation and identification of conventional bacteria involved in the pathological process, according to current bacteriological techniques recommended by Quinn et al. (1994).

Viruses and *Mycoplasma* were excluded from the search because they require specialized culture conditions and identification techniques. In the laboratory, the samples were thawed at ambient temperature. The surface of the lung fragment was cauterized on the surface; the parenchyma was cored with an unbuttoned Pasteur pipette and placed in nutrient enrichment broth (manufactured by Pasteur Institute of Algeria, IPA) to revitalize bacteria (37°C for 18 to 24 h), while the swabs were directly and separately placed in similar broths.

The enriched broths were seeded on agar with 5% sheep blood (IPA) incubated under aerobic conditions at 37°C for 24 h. Morphological characteristics of colonies were scored on blood agar, as well as the presence or absence of hemolysis, the type of this latter and the production of pigments and smells. After purification, Gram stain, catalase and oxidase tests were performed. The choice of the identification gallery was established from the results of these tests.

RESULTS

Prevalence of pulmonary lesions

From 1018 slaughtered and examined sheep during the survey, 194 (19%) had pulmonary lesions, isolated or in combination, of varying nature and severity. The lesions appearing to be infectious (n = 75) were sampled. The rest of the observed lesions (n=119) were either parasitic in nature or slaughter injuries and were not taken into account for the result of the investigation.

Distribution of the lesion types

Infectious pulmonary lesions can be divided into 4 categories: red hepatization, grey hepatization, atelectasis and emphysema. Pulmonary hepatization (also called consolidation injury) represented the most frequent lesion observed in the slaughter house; all forms included, it represents 70.7% of the lesions (Table 1). Considering that different lesions sometimes occur in the same lung, only the dominant one was considered.

Location of lesions

Pulmonary lesions mostly sits on the apical lobes, the right one in particular (88% of the inspected lungs), reaching either part or all of these, and sometimes extends to other lobes (Table 2). Lesion frequency is higher on the right side of the lung, with a marked laterality for apical lobes (Figure 1).

Scope of lung injury

Determining the extent of the pulmonary lesion on each lobe permits us to estimate the evolution of the pathology. The scale of lung lesions depends on the severity and extension rate of pneumonia (Table 3). We note that the apical right lobe is frequently affected in its entirety.

Lesion score

Lesions extending on small areas are the most prevailing; three quarters of the examined lungs showed a lesion

Table 2. Distribution of pulmonary lesions observed in the lobes.

Lung	Lung lobes (n=75)	Number	%
Right Lung	Right apical lobe	66	88
	Anterior medium lobe	14	19
	Posterieur medium lobe	08	11
	Right caudal lobe	07	09
	Azygos lobe	03	04
Left Lung	Left apical lobe	13	17
	Left medium lobe	09	12
	Left caudal lobe	04	05

Figure 1. Total red hepatization (or consolidation) of the apical and medium lobes of the lung, taking the color and consistency of the liver (dorsal view).

score smaller than 5/32. The average lung score was equal to 5.26 ± 5.22, while the number of animal per injured lobes was 1.82±1.63. Lung lesions extend widely over the different lobes. The perimeter areas where pneumonia is evolving are irregular.

Bacteriological analysis results

Bacteriological analysis was performed on 150 samples from 75 animals. The samples were 75 fragments of lung and 75 bronchial swabs of the same lungs with lesions. The most frequently isolated bacteria were gamma-hemolytic streptococci (18%), *Escherichia coli* (17.7%), *Micrococcus* species (14.5%) and coagulase-negative staphylococci (10.4%). The family of Enterobacteriaceae (*E. coli* included) represents nearly 43% of the isolates. *M. haemolytica* and *P. multocida* account for 5.6% of isolates.

Most bacteria isolated (71%) were either saprophytes or commensals of the upper respiratory system and represent commonplace bacteria contamination (*Bacillus*, *Proteus*, *Micrococcus*). Others (*E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas*, *Streptococcus* βeta-hemolytics) which account for 23%, are occasional specific pathogenic agents of the respiratory system, and may be pathogenic in secondary infections due to a decrease in immune defenses of the host. The role of the *M. haemolytica* and *P. multocida* (5.6%) is well known in respiratory diseases in sheep (pneumotropic bacteria) (Figure 2).

Several associations of bacterial species were isolated in the same sample (Figure 3). Out of the 150 samples (75 swabs and 75 lung parenchyma), 54 (36%) were mono-microbial, 87 (58%) indicated the association of two bacterial species and 9 (6%) showed three. All samples showed bacterial growth; none were sterile. This combination of bacteria in one sample reflects a multifactorial etiology of lung disease of sheep and explains the diversity of the observed lesions. Comparison of the results obtained by the two samples types highlights a greater number of bacterial flora, from the swabs.

According to these results, there seems to be no relationship between bacterial groups and sample type (Figure 4). Pneumotropic bacteria were isolated only in hepatization lesions (Table 4).

Despite the small number of pneumotropic bacteria (*M. haemolytica* and *P. multocida*) isolated in this study, their exclusive frequency (14/14) should be noted in the consolidation lesions (hepatization).

DISCUSSION

Sheep farming in Algeria represents the main source of income for many families in rural areas. Ouled Djellal sheep are the most common breed of sheep, accounting for more than 60% of the national herd. They provide a lot of meat, wool and leather, and are particularly suited to the arid climate. However, the important variations in temperature, ranging from hot in summer to very cold in winter, can promote the development of sheep respiratory diseases. Indeed, previous studies in France (Douart, 2002), Algeria (Belkhiri et al., 2008) and India

Table 3. Extent of lung lesions in sheep observed at the abattoir.

Extent (%)	Number of sheep (n=75)							
	Left Lung (3 lobes)			Right Lung (5 lobes)				
	Apical	Medium	Caudal	Apical	Anterior medium	Posterior medium	Caudal	Azygos
0	62	65	70	07	58	64	63	73
< 25	05	02	01	12	01	03	04	01
25-50	05	05	00	13	08	03	00	00
50-75	00	00	00	03	00	00	00	00
75-100	01	00	00	09	02	00	00	00
100	02	03	04	31	06	05	08	01

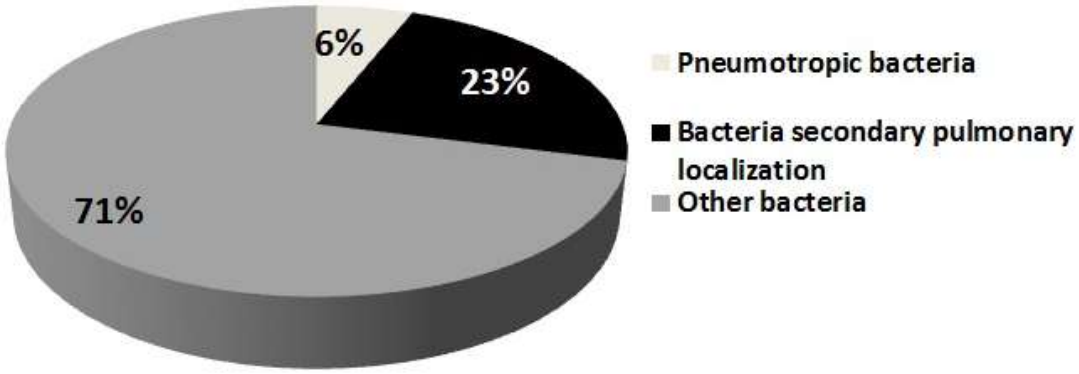

Figure 2. The prevalence of pathogenic bacteria isolated from pneumonic lungs of sheep slaughtered.

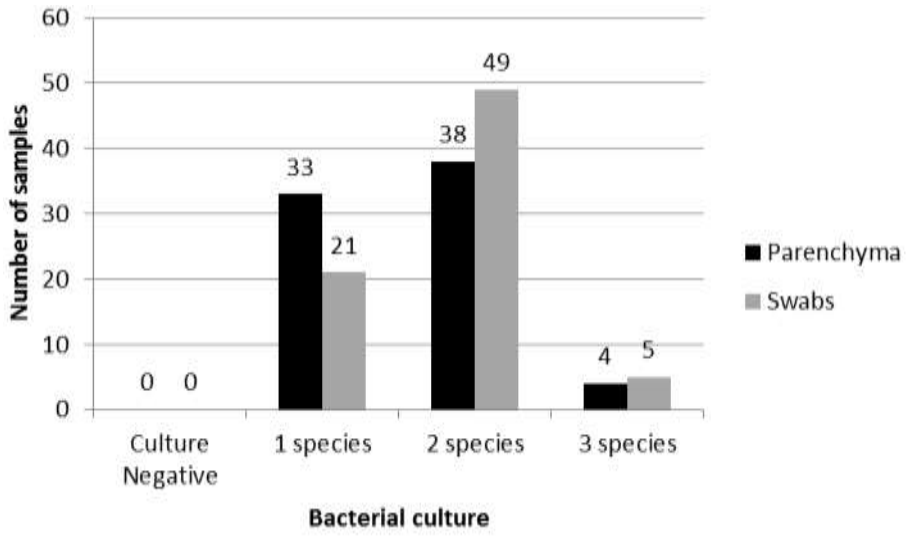

Figure 3. Bacterial associations by sampling procedure .

(Dar et al., 2012) have determined a significant incidence of lung lesion in sheep during winter. In regard to the unavailability of some laboratory techniques, the results of the investigation lack precision, particularly because of the difficulty to look for *Mycoplasma* and viruses, which are much involved in lung lesions in sheep.

The molecular diagnosis of *Pasteurella* spp. isolates

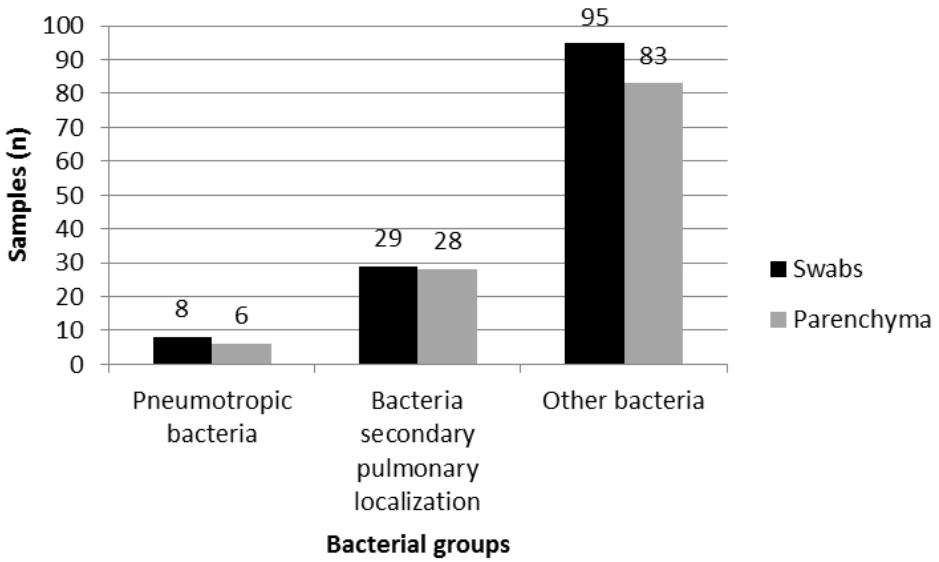

Figure 4. Distribution of bacterial groups in sheep showing lung lesions according to the sample type.

Table 4. Relationship between macroscopic lesions and the isolated bacteria of the lungs.

Lesion	Bacteria								
	Pneumotropic bacteria			Bacteria secondary pulmonary localization					Other*
	M.h.	P. m.	Past. spp	*E. coli*	SCP	Klebs. pneu.	Pseudo.	Strept. β-hemo.	
Red hepatization	**5**	**6**	2	21	6	0	1	1	93
Grey hepatization	0	3	1	8	1	0	0	0	30
Atelectasis	0	0	0	2	0	1	0	0	28
Emphysema	0	0	1	10	1	1	0	0	14
Other**	0	0	0	0	0	0	0	0	13
Total	5	9	4	41	8	2	1	1	**178**

M.h : *Mannheimia haemolytica*; P.m: *Pasteurella multocida*; Past. spp: *Pasteurella* spp.; SCP: coagulase-positive Staphylococci; Klebs. Pneu: *Klebsiella pneumoniae* ; Pseudo.: *Pseudomonas,* Strept. β-hémo.*:* beta-hemolytic Streptococci. Other bacteria*: Commonplace bacteria contamination (*Bacillus*, *Proteus*, *Micrococcus*…). Other lesions**: Congestion, Abscesses …

which includes analysis of nucleotide sequences of the 16S rRNA and *KMT1* genes overcame the disadvantage and limitation of phenotypic diagnosis and became a favorable technique in many international laboratories for identification and calculating the phylogenetic tree in Pasteurellaceae family. It reduces the duration of identification, allows a direct detection of organisms from clinical sample's genome, and enhances the sensitivity and specificity of the diagnosis (Hassan et al., 2016).

Polymerase Chain Reaction (PCR) is a sensitive and rapid diagnostic procedure for the early diagnosis of *Mycoplasma* infection. *M. ovipneumoniae* has been isolated and detected through PCR from the nasal swab samples and from pneumonic sheep lungs (Amin et al., 2016).

In Algeria, Kabouia (2005) highlighted the association of *Mycoplasma* with different types of lung lesions in 21% of sheep samples. *Mycoplasma capricolum* alone or in combination with other mycoplasmas (*Mycoplasma agalactiae* and *M. ovipneumoniae*, *Mycoplasma arginini*) and other bacteria are responsible for ovine respiratory diseases. In Richard et al. (1986) in France, isolated *M. ovipneumoniae* in more than half of pneumonia lesion samples from sheep.

Statistically highly significant difference (Fisher's exact test $P \leq 0.0001$) in the isolation of *M. ovipneumoniae* between healthy and respiratory distressed sheep breeds in Balochistan (Pakistan) was found (Amin et al., 2016). The sampling is not random because it is not based on any lot. It is not representative of the ovine population, at least that of the Blida region. Despite this lack of representativeness, the results identified in this study

make it possible to learn about relationships that may exist between the lesions and pneumotropic bacteria. The selected animals cannot be considered as representative of the sheep population of the study area, given that the only criterion for inclusion is that the animal, regardless of age or sex, arriving at the slaughterhouse showed macroscopic lung lesions, infectious and non-parasitic. Moreover, we had no information about their mode of rearing or any recent antibiotic treatments. The examined animals were all males, 6 to 12 months. For practical reasons, the samples had to be frozen pending their exploitation, despite the fact that some works showed a deleterious effect of freezing on the number of species isolated in the pulmonary bacteriology (Menoueri, 1985; Cadoz, 2000). The effect of freezing can be ambivalent; it can be negative by reducing the probability to isolate *Pasteurella* and even completely eliminate it, including the pneumonia principal agent; on the other hand, it can be beneficial by preventing the development of bacteria that proliferate in the post mortem process.

Our study showed a prevalence of 19% lung lesions. In the large scale study of Goodwin-Ray et al. (2008) carried out on about 2 million lambs slaughtered in three abattoirs in New Zealand, the prevalence of pneumonia ranged from 7 to 13% per slaughter house. An abattoir survey realized by Brunet and Fontaine (1980) in France showed that on more than 25,000 inspected lambs, 35% had pulmonary lesions. In other countries, the prevalence of pneumonia in not frozen samples was found as follows: 15.28, 22.48 and 18.93% in India, respectively in 2013, 2014 and 2016 (Priyadarshi et al., 2013; Asok et al., 2014; Amaravathi et al., 2016); 7.8% in Tanzania (Mellau et al., 2010); 4.2% in Iran (Azizi et al., 2013), 27.84% in Algeria (Belkhiri et al., 2014). Pneumonia lesions observed in our study, affected preferentially apical lobes (88% of the cases). The predominance of the lesions in cranioventral portions of lung has been attributed to shortness and abrupt branching of air ways, greater deposition of infectious organisms, inadequate defense mechanisms, reduced vascular perfusion, gravitational sedimentation of exudates and regional differences in ventilation (Dar et al., 2013).

In most domestic animal species, the right cranial lobe is ventilated down by the cranial bronchus, with the exception of ruminants and pigs lobe where it is ventilated by an additional bronchus which lies just before the tracheal bifurcation (Nickel et al., 1973). The higher frequency of lesions on the right side of the lung and the increased vulnerability of apical and cardiac lobes are similar to those described by Alley (1975), Haziroglu (1994), Ozyildiz et al. (2012) and Dar et al. (2014); this is a special form of pneumonia called chronic or atypical pneumonia.

Bacteriological results showed a diverse bacterial flora in the lungs as well as in the bronchial liquid, predominantly gram positive, similar to the results of Garedew et al. (2004) in Ethiopia. The results indicated in this study, including the relatively small number (5.6%) of *Pasteurella* isolation are to be kept with a sense of proportion. It is possible that these results were due to the fragility of the bacteria that have not withstood the samples storage conditions. As concerns in pneumonic lungs, the results of isolates of *M. haemolytica* (without freezing) were the following: 21. 96% (Marru et al., 2013), 34.1% (Demissie et al., 2014), and 14.7% (Asaye et al., 2015) in Ethiopia; 22.20% (Kaoud et al., 2010) and 14.3% (Saed et al., 2015) in Egypt, respectively.

According to Cadoz (2000), *M. haemolytica* was isolated twice as often when there was no freezing, while most of the bacteria resist, at least partially, freezing. According to Meyer et al. (2004), freezing has no real bactericidal effect; for the most sensitive microorganisms (gram-negative), the population is reduced by a power of 10 by freezing (that is 90% destroyed) and again the power of 10 during prolonged storage. If the population is large at the time of freezing, it will be so after storage and thawing.

In their study, Tehrani et al. (2004) attributed *M. haemolytica* low isolation either to antibiotic treatment or to the development of other bacteria such as *Proteus* species and *Bacillus* species which would mask the presence of *Pasteurella* or chronic lesions that would promote growth of other bacteria. Bacteria similar to those identified in our study were isolated from sheep with pneumonia. This is the case of Richard et al. (1986) in France; Al Sultan (1995) in Iraq; Barbour et al. (1997) in Saudi Arabia and Yimer and Asseged (2007) in Ethiopia, with substantially similar proportions. The distribution of different bacterial groups is relatively similar to that found by Menoueri (1985) in France on housed lambs with the difference that he studied in addition with the presence of *Mycoplasma* spp. (12%).

The isolated bacteria from the diseased lungs of the animals studied were dominated by streptococci gamma-hemolytic and *E. coli* which are opportunistic pathogens and develop in parallel to primary pathogens during an infectious phenomenon (Richard et al., 1986). Streptococci were isolated in 18% of the cases. They are part of the normal flora of the upper respiratory tract and are considered opportunistic pathogens (Kaoud et al., 2010). *Escherichia coli* is extremely widespread in the environment, developing more whenever there is contamination of the sample. Its presence in the lungs can possibly result from the evolution of septic processes. In our study, the predominance of *E. coli* was also highlighted by Mohammed (1999). Robbins et al. (1981) reported that *S. aureus* colonizes the upper respiratory tract, become involved in the disease process when stress conditions prevail.

According to Richard et al. (1986), the greater concentration of the bacterial flora obtained from the swabs (132 species isolated against 117 for parenchyma) could be explained by the presence of bacteria in the

open air, which are absent from the lung parenchyma or contamination during handling. Swabs appear much more polluted with contaminants such as the common *Micrococcus, Proteus* and *Bacillus.* The isolation of *M. haemolytica* and/or *P. multocida*, specifically the correlation between these bacteria and lung injury called consolidation (hépatization) corroborate the findings of Sharp (1978), Jones et al. (1982), Pfeffer (1983) or Daniel et al. (2006).

For Cadoz (2000), the involvement of *M. haemolytica* increases with further damages. According to Garedew (2004), a strong correlation was observed between the presence of *Pasteurella* and the development of pneumonia in sheep. Moreover, according to Kaoud et al. (2010), *M. haemolytica* plays a more important role in respiratory diseases of sheep and goats (14%) than in cattle (3.6%).

Our study aimed to determine the situation of sheep, sacrificed at the slaughterhouse of Blida, vis-à-vis respiratory diseases based on pathological and bacteriological investigations. The infection rate found reached the considerable figure of 20% of the total slaughtered animals. Bacteriological results showed the presence of a wide variety of bacteria, including pneumotropic germs (*M. haemolytica* and *P. multocida*), isolated up to 5.6% and strongly correlated to hepatization which represents 70% of lesions on lungs. The low percentage of isolation of these bacteria is probably due to the freezing of the samples. These germs caused pneumonia in young animals (under one year), primarily localized on the apical right lobe (88% of cases). It was also noted that the nature of isolated germs is independent from the sample type. These results reflect the existence of atypical pneumonia in sheep populations in Algeria; this pathology should be taken into consideration, because of its harmful impact on the health and growth performance of young animals. Further studies aiming to know the different serotypes of these agents that prevail in the country will allow the development of a vaccine. Research of *Mycoplasma* and viruses, including parainfluenza, which are very involved in respiratory diseases, is strongly recommended too.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Abdelguerfi A, Ramdane SA (2003). Bilans des Expertises sur La Biodiversité importante pour l'agriculture en Algérie. MATE-GEF/PNUD.Projet ALG/97/G31, 231.

Alley MR (1975). The bacterial flora of the respiratory tract of normal and pneumonic sheep. New Zealand Vet. J. 23(6):113-118.

Al Sultan II (1995). Bacterial isolation from pneumonic lungs in sheep. Iraqi J. Vet. Sci. 8:213-215.

Amaravathi M, Satheesh K, Annapurna P, Subramanyam KV (2016). Incidence of spontaneous lung lesions in slaughtered sheep. Int. J. Adv. Res. Biol. Sci. 3(2):269-271.

Amin S, Rahman S, Hussain I, Muhammad G, Awan MA, Ahmad Z, Tariq MM, Ullah MI, Kakar MA (2016). Bimolecular identification of *M.ovinpeumoniae* from nasal swab samples of sheep from various districts of Balochistan, Pakistan. Int. J. Adv. Biol. Biomed. Res. 4(1):32-39.

Asaye M, Biyazen H, Bezie M (2015). Isolation and Characterization of Respiratory Tract Bacterial Species from Domestic Animals with Pneumonic Lungs from Elphora Abattoir, Ethiopia. Int. J. Microbiol. Res. 6(1):13-19.

Asok Kumar M, Kumar R, Varshney KC, Nair MG, Lakkawar AW, Sridhar BG, Palanivelu M (2014). Pathomorphological studies of lung lesions in sheep. Indian J. Vet. Pathol. 38(2):75-81.

Azizi S, Korani FS, Oryan A (2013). Pneumonia in slaughtered sheep in south-western Iran: pathological characteristics and aerobic bacterial aetiology. Vet. Italiana 49(1):109-118.

Barbour EK, Nabbut NH, Hamadeh SK, Al Nakhli HM (1997).Bacterial identity and characteristics in healthy and unhealthy respiratory tracts of sheep and calves. Vet. Res. Commun. 21:421-430.

Belkhiri M, Tlidjane M, Meziane T (2008). Fréquence des lésions pulmonaires des bovins et ovins de Tiaret et Batna. Rencontre autour des Recherches sur les Ruminants 15, 68 p.

Belkhiri M, Benhathat Y, Tlidjane M (2014). Classification and frequency of ovine pulmonary lesions in Tiaret's slaughterhouse. Res. J. Pharma. Biol. Chem. Sci. 5(2):1181-1188.

Boutonnet JP (1998). Spécificité et diversité des filières viandes rouges en Méditerranée, CIHEAM- Options Méditerranéennes, pp. 31-37.

Brunet J, Fontaine M (1980). Essai d'estimation des pertes par maladies dans le troupeau ovin du sud-est de la France à travers une enquête en abattoir. Bulletin de l'Office International des Epizooties 92:355-369.

Cadoz MO (2000). Contribution à l'étude des broncho-pneumonies du chamois. Analyse de 368 rapports d'autopsie et examens bactériologiques. Thèse de doctorat vétérinaire, ENV Lyon. Thèse n ° pp. 81-144.

Chakraborty S, Kumar A, Tiwari R, Rahal A, Malik Y, Dhama K, Pal A, Prasad M (2014). Advances in Diagnosis of Respiratory Diseases of Small Ruminants. Veterinary Medicine International, Article ID 508304, 16 p

Cutlip RC, Lehmkuhl HD, Brogden KA (1993). Chronic effects of coinfection in lambs with parainfluenza-3 virus and *Pasteurella haemolytica*. Small Rum. Res. 11:171-178.

Daniel JA, Held JE, Brake DG, Wulf DM, Epperson WB (2006). Evaluation of the prevalence and onset of lung lesions and their impact on growth of lambs. Am. J. Vet. Res. 67:890-894.

Dar LM, Darzi MM, Mir MS, Rashid A, Abdullah S, Hussain SA (2012). Prevalence and pathological studies on ovine pneumonic pasteurellosis in Kashmir valley, India. Eur. J. Vet. Sci. 28(4):199-203.

Dar LM, Darzi MM, Mir MS, Kamil SA, Rashid A, Abdullah S (2013). Prevalence of lung affections in sheep in northern temperate regions of India: A postmortem study. Small Rum. Res. 110:57-61.

Dar LM, Darzi MM, Mir MS, Kamil SA, Rashid A, Abdullah S, Hussain SA, Rather FA, Parihar S (2014). Histopathological and histoenzymatic studies on bronchopneumonia in sheep .J. Appl. Animal Res. 42(3):289-296.

Demissie T, Dawo F, Sisay T (2014). Biochemical and Antigenic Characterization of *Mannheimia*, *pasteurella* and *Mycoplasma* Species from Naturally Infected Pneumonic Sheep and Goats, Bishoftu, Ethiopia. Afr. J. Basic & Applied Sciences 6(6):198-204.

Douart A (2002). Les pasteurelloses des petits ruminants. Le Point Vétérinaire 33:86-89.

Galapero J, Fernández S, Pérez CJ, Calle-Alonso F, Rey J, Gómez L (2016). Identifying risk factors for ovine respiratory processes by using Bayesian networks. Small Rum. Res. 136:113-120.

Garedew L, Avelet G, Yilma R, Zeleke A, Gelaye E (2004). Isolation of diverse bacterial species associated with visna-maedi infection of sheep in Ethiopia. Afr. J. Microbiol. Res. 4(1):14-21.

Giangaspero M, Vanopdenbosch E, Nishikawa H, Tabbaa D (1997). Prevalence of antibodies against respiratory viruses (Parainfluenza virus type 3, respiratory syncytial virus, reovirus and adenovirus) in

relation to productivity in Syrian Awassi sheep. Trop. Anim. Health Prod. 29:83-91.
Goodwin KA, Jackson R, Brown C, Davies PR, Morris RS, Perkins NR (2004). Pneumonic lesions in lambs in New Zealand: patterns of prevalence and effects on production. New Zealand Veterinary Journal 52:175-179.
Goodwin-Ray KA, Stevenson M, Heuer C, Pinchbeck G (2008).Hierarchical and spatial analyses of pneumonia-lesion prevalence at slaughter in New Zealand lambs. Preventive Vet. Med. 83:144-155.
Hassan GM, El-Feky ZA, Eissa EA, Teleb AA (2016). Rapid diagnosis of virulent *Pasteurella multocida* isolated from farm animals with clinical manifestation of pneumonia respiratory infection using 16S rDNA and *KMT1* gene. Asian Pac. J. Trop. Dis. 6(1):21-26.
Haziroglu R, Diker KS, Gulbahar MY, Akan M, Guvenc T (1994). Studies of the pathology and microbiology of pneumonic lungs of lambs. Deutsche Tierarztliche Wochenschrift 101:441-443.
Jones GE, Field AC, Gilmour JS, Rae AG, Nettleton PF, McLauchlan M (1982). Effects of experimental chronic pneumonia on bodyweight, feed intake and carcasse composition. Veterinary Record 110:268-273.
Kaoud H, El Dahshan AR, Zaki MM, Shimaa AN (2010). Occurrence of *Mannheimia haemolytica* and *Pasteurella trehalosi* among ruminants in Egypt. N. York Sc. J. 3(5):135-141.
Kabouia R (2005). Etude épidémiologique des mycoplasmes des petits ruminants. Application de l'immunoblot à l'étude des sérums de moutons infectés expérimentalement par *Mycoplasma agalactiae*. Thèse de Doctorat d'Etat, Université des frères Mentouri, Constantine, Institut des sciences vétérinaires, P 116.
Lemhkuhl HD, Cutlip RC, Bolin SR, Brogden KA (1985). Seroepidemiologic survey for antibodies to selected viruses in the respiratory tract of lambs. Am. J. Vet. Res. 46:2601-2603.
Loosli CG (1968). Synergism between respiratory viruses and bacteria. Yale J. Biol. Med. 40:522-540.
McIlroy SG, Goodall EA, McCracken RM, Stewart DA (1989). Rain and wind chill as factors in the occurrence of pneumonia in sheep. Vet. Rec. 125:79-92.
MADR (Ministry of Agriculture and Rural Development) (2014). Agricultural Statistics (areas and productions serie B,). Algiers.
Malone FE, McCullough SJ, McLoughlin MF, Ball HJ, O'Hagan J, Neill SD (1988). Infectious agents in respiratory disease of housed fattening lambs in Northern Ireland. Vet. Record 122(9):203-207.
Marru HD, Anijajo TT, Hassen AA (2013). A study on Ovine pneumonic pasteurellosis: Isolation and Identification of *Pasteurellae* and their antibiogram susceptibility pattern in Haramaya District, Eastern Hararghe, Ethiopia. BMC Vet. Res. 9:239.
Martin WB (1996). Respiratory infections of sheep. Comparative Immunol. Microbiol. Infect. Dis. 19(3):171-179.
Mellau LSB, Nonga HE, Karimuribo ED (2010). A slaughterhouse survey of lung lesions in slaughtered stocks at Arusha, Tanzania. Preventive Vet. Med. 97:77-82
Menoueri MN (1985). Ecopathologie des pneumopathies ovines. Contribution à l'étude des affections pulmonaires chez les agneaux de bergerie. Maîtrise Ès sciences vétérinaires, ENV Lyon, P 66.
Meyer A, Deiana J, Bernard A (2004). Cours de microbiologie générale: avec problèmes et exercices corrigés. Edition Doin. P 430.
Mohammed R (1999). Bacteriological and histological examinations of pneumonic lungs of small ruminants slaughtered at Hashime Nure export abattoir. Debre Zeit. Ethiopia DVM thesis, AAU, FVM. Debre Zeit, Ethiopia, pp. 1-21.
Nickel R, Schummer A, Seiferle E (1973). Respiratory System, The viscera of the domestics animals. New York, NY, Springer-Verlag, P 245.
Ozyildiz Z, Tel OY, Yilmaz R, Ozsoy SY, Keskin O (2013). Pathological and Microbiological Investigations of Pneumonic Pasteurellosis in Sheep. Kafkas Üniversitesi Veteriner Fakültesi *D*ergisi 19(1):103-108.
Pfeffer A, Thurley DC, Boyes BW, Davies GB, Price MC (1983). The prevalence and microbiology of pneumonia in a flock of lambs. New Zealand Veterinary J. 31(11):196-202.
Priyadarshi BH, Joshi DV, Patel BJ,Raval SH, Patel HA (2013). Pathomorphology of spontaneously occurring pulmonary lesions in sheep (*Ovis aries*). Rum. Sci. 2(1):31-35.
Quinn PJ, Carter ME, Markey B, Carter GE (1994). Bacterial pathogens: Microscopy, culture and identification. In: Clinical Veterinary Microbiology. Wolfe Publishing, London, pp. 21-60.
Richard Y, Menoueri MN, Guigen F, Favier C, Borges E, Fontaine M, Oudar J., Brunet J, Pailhac C (1986). Pneumopathies de l'agneau de bergerie. Etude bactériologique sur des poumons prélevés à l'abattoir. Rev. Méd. Vét. 137(10):671-680.
Robbins SL, Angell M, Kumar U (1981) The respiratory system in basic pathology. 3rd edition, Philadelphia, PA, USA, WB Saunders, pp. 369-420.
Rouchy S (1992). Diagnostic différentiel des affections respiratoires chez les ovins. Thèse pour le doctorat vétérinaire, ENV Alfort, p88.
Saed M, Khaliel S, Abd El-Mageed A, Khalifa E (2015). Genome Sequences of Gcp Gene of *Mannheimia haemolytica* Serotypes A1 and A2 Associated with Respiratory Manifestation of Ruminant in Egypt. Global Vet. 14 (1):142-148.
Sharma R, Woldehiwet Z (1990). Increased susceptibility to *Pasteurella haemolytica* in lambs infected with bovine respiratory syncytial virus. J. Comp. Pathol. 103:411-420.
Sharp JM, Gilmour NJ, Thompson DA, Rushton B (1978). Experimental infection of specific pathogen-free lambs with parainfluenza virus type 3 and *Pasteurella haemolytica*. J. Comp. Pathol. 88:237-243.
Tehrani AA, Ras MB, Niazy H (2004). Isolation and identification of *Pasteurella haemolytica* biotype A from sheep in Urmia, Iran. Iranian Journal of Veterinary Research, University of Shiraz. 5(2):1383, 105-109.
Valadan M, Jabbari AR, Niroumand M, Tahamtan Y, Bani Hashemi SR (2014). Isolation and Identification of *Pasteurella multocida* from Sheep & Goat in Iran. Arch. Razi Inst. 69(1):47-55.
Yimer N, Asseged B (2007). Aerobic bacteria flora of the respiratory tract of healthy sheep slaughtered in Dessie municipal abattoir, north eastern Ethiopia. Rev. Méd. Vét. 158(10):473-478.
Zrelli M, Haddad N, Bouzouaia M (1988). La Pasteurellose. In : Fassi-Fehri M, Les maladies infectieuses du mouton. Tome 1, Actes éditions, pp. 206-229.

academicJournals

Vol. 10(40), pp. 1694-1702, 28 October, 2016
DOI: 10.5897/AJMR2016.8092
Article Number: 4A402F961332
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

External ocular bacterial infections among Sudanese children at Khartoum State, Sudan

Mazin O. Mohager[1]*, Lemya A. Kaddam[2] and Samah O. Mohager[3]

[1]Department of Pathology, College of Medicine, Al-Jouf University, Skaka, Kingdom of Saudi Arabia.
[2]Department Clinical laboratory Science, College of Applied Medical Sciences, Al-Jouf University, Skaka, Kingdom of Saudi Arabia.
[3]Unit of Immunology, School of Medicine, Ahfad University of Women, Omderman, Sudan.

Received 5 May, 2016; Accepted 15 September, 2016

Ocular infections are widespread and they exert heavy burden on eye health. Virtually, any eye component can be infected by a diversity of bacteria. The present study was performed to determine the prevalence of external ocular bacterial infections and to find out antibiotic susceptibility pattern of bacterial isolates at eye care hospitals in Khartoum, Sudan. Two hundred and four corneal scrape and drained pus materials were received from infected eyes with clinical diagnoses of bacterial conjunctivitis, keratoconjunctivitis, keratitis, blepharo-conjunctivitis, blepharitis, dacryo-cystitis and eye abscess. Culture, microscopy with Gram's stain of both samples, bacterial colonies and biochemical tests were carried out. Antibiotic susceptibility analysis using Kirby–Bauer disc diffusion test and standard table of antibiotic susceptibility was performed. Out of 204 samples processed, 130 (63.7%) yielded bacterial growth. The most prevalent bacterial eye infection was conjunctivitis (59.2%). Of all the isolates, 75 (57.7%) were Gram's positive and 55 (42.3%) were Gram's negative. Coagulase positive *Staphylococcus aureus* were the most prevalent, 39 (30%) followed *by Streptococcus pneumonia* 31 (23.8%), *Haemophilus influenzae* 22 (16.9%), *Pseudomonas aeruginosa* 13 (10%) and *Neisseria gonorrheae* 10 (7.7%). Gram positive bacteria were highly sensitive to vancomycin (95%), followed by chloramphenicol and ciprofloxacin (91%) and ceftriaxone (84%), while the majority were resistant to penicillin (72%). Gram-negative organisms were highly susceptible to amikacin (92.7%) followed by ceftriaxone (87.3%) and ciprofloxacin (78.2%). Major resistance was towards cotrimoxazole (82%) and ampicillin (73%).

Key words: External ocular bacterial infections, bacterial isolate, antibiotic susceptibility, normal flora, pathogenic bacteria.

INTRODUCTION

Eye is protected by epithelia and mucous membranes which both serve as mechanical, chemical and biological barriers against pathogens (Bolaños-Jiménez et al., 2015). Tearing is an innate immunity mechanism that

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

flushes foreign particles from the ocular surface and act as a transport vehicle for the transfer of antimicrobial proteins and immunoglobulins (Akpek and Gottsch, 2003).

Like other mucosal surfaces, eyes are covered with protective resident microbial flora (Linden et al., 2008). Normal microbial flora of eye secretes antibacterial substances and competes with pathogenic bacteria for site and nutrients (Venkataraman et al., 2015).

Micro-organisms such as viruses, bacteria, unicellular parasites and fungi as well as multicellular parasites are capable of attacking both the surface and the interior of the eye giving rise to ocular diseases. This is the outcome of the interplay between invading organism's strong virulence factors and the host's depressed resistance. Poor personal hygiene, unfortunate living conditions, low socio-economic class and decreased immune status can lead to depressed host resistance (Tesfaye et al., 2013). Bacteria are the most frequent causative agents of ocular infections that might possibly culminate in loss of vision and this justifies the need for prompt treatment of serious bacterial eye infections that threatens the eye (Ubani, 2009).

Ocular infections include conjunctivitis and keratitis. These infections can damage structures of the eyes if left untreated, leading to considerable disabilities including blindness (Ubani, 2009). Other eye infections encompass dacryocystitis, dacryoadenitis, cellulitis and eye abscess (Brissette et al., 2011). Bacterial blepharitis represents another important ophthalmic infection (Bertino, 2009).

The most common worldwide mild eye infections detected in primary care clinic is conjunctivitis (Hovding, 2008). Bacterial conjunctivitis, or red eye, involves inflammation of the conjunctival mucosa. According to the American Academy of Ophthalmology Cornea and External Disease, this condition is more common in young children and the elderly than in other age groups (American Academy of Ophthalmology Cornea, 2011). The most common aetiological pathogens in bacterial conjunctivitis are *Staphylococcus aureus*, *Streptococcus pneumonia*, *Haemophilus influenzae*, *Staphylococcus epidermidis*, *Enterococcus* spp., *Moraxella* spp., *Streptococci viridans* group, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Proteus mirabilis* (Bartlett and Jaanus, 2008; Cavuoto et al., 2008). Staphylococcal infections predominate in adults while *S. pneumoniae* and *H. influenzae* are more prevalent in children (Cavuoto et al., 2008).

Bacterial keratitis is another entity of ocular infections that might follow corneal epithelial barrier disruption due to injury or trauma with subsequent ulceration and infiltration of inflammatory cells (Kaliamurthy et al., 2013). The usual organisms responsible for such infections include Gram-positive bacteria such as *S. aureus, S. epidermidis* and several *Streptococcus* and *Bacillus* spp. as well as Gram-negative bacteria like *P. aeruginosa, Moraxella* spp. and *Haemophilus* spp. Corneal scarring or perforations are possible devastating outcomes, the avoidance of which necessitates immediate diagnosis and treatment (Rahimi et al., 2015).

Bacterial blepharitis is an infection of the eyelid margin with subsequent engorgement, congestion and eyelashes crusting (Rahimi et al., 2015). Bacterial blepharitis is mostly caused by *S. aureus* and coagulase negative staphylococci (CoNS) (Musa et al., 2014).

Dacryocystitis is painful inflammation of the lacrimal sac. Obstruction of the nasolacrimal duct, whether congenital or acquired is a known predisposing factor (Rahimi et al., 2015). The most common isolates in dacryocystitis are P*. aeruginosa, S. aureus, Enterobacter aerogenes, Citrobacter, S. pneumoniae, E. coli* and *Enterococcus* spp. (Briscoe et al., 2005; Kubal and Garibaldi, 2008).

Dacryoadenitis is a related condition in which there is inflammation and infection of the lacrimal gland. It can be caused by a variety of bacterial agents including *S. aureus, Neisseria gonorrheae* and streptococci. Clinically, it causes pain, redness, swelling, tearing and discharge over the lacrimal gland (the lateral one-third of the upper eyelid) (Brissette et al., 2011).

Cellulitis is bacterial infection of the periocular tissue. The condition can be serious to the extent of causing vision loss (Stratton et al., 2015). It can be classified as preseptal cellulitis, orbital cellulitis, subperiosteal abscess, intraorbital abscess and cavernous sinus thrombosis related cellulitis (Gonzalez and Durairaj, 2010). Preseptal cellulitis (PC) is defined as an inflammation of the eyelid and surrounding skin with eyelid abscess being a possible outcome (Akçay et al., 2014). The most frequent pathogen implicated in the etiology of this group of ocular infections is *H. influenza* (Stratton et al., 2015). Currently, *S. aureus* and *Streptococcus* species cause the majority of culture positive cases of preseptal cellulitis (Gonzalez and Durairaj, 2010). This is particularly noticeable when there are co-existing local wounds (Stratton et al., 2015).

Regardless of the predisposing factor beyond eye lid abscess, local skin flora such as *S. aureus* is the classical causative agents (Rutar et al., 2005).

Specific antibacterial agents are the corner stone in the management of bacterial ocular infections. Defining the specific antibacterial drug requires isolation and identification of bacterial pathogens along with antibiotics susceptibility analysis (Sharma, 2011). The empirical choice of an effective treatment is becoming more difficult as ocular pathogens are increasingly becoming resistant to commonly used antibiotics (Khosravi et al., 2007). Bacterial resistance is influenced by pathogens characters and antibiotic-prescribing practices including the widespread use of systemic antibiotics together with the applied health care guidelines (Bertino, 2009).

The present study aimed at determining bacterial isolates of external eye infections among Sudanese children patients at Khartoum state in addition to studying

the distribution of the common bacterial isolates in the specific clinical entities of bacterial conjunctivitis, kerato-conjunctivitis, keratitis, blepharo-conjunctivitis, blepharitis, dacryo-cystitis and eye abscess. It also worked towards finding out the distribution of these bacterial isolates among age groups and gender as well as assessing the *in vitro* susceptibility of these ocular bacterial isolates to the commonly used antibiotics in Sudan.

MATERIALS AND METHODS

Study design

This is a cross-sectional study that included patients with clinically diagnosed bacterial conjunctivitis, keratoconjunctivitis, keratitis, blepharo-conjunctivitis, blepharitis, dacryo-cystitis and eye abscess. All patients were diagnosed by a number of ophthalmologists using standard protocols.

Ethical consideration

The study was approved by the National Ethical Committee, Ministry of Health Sudan. Permissions were taken from all hospitals administrations which were involved in this study. Written consents were obtained from every participant when applicable, or their caretakers before the enrollment in the study.

Study samples

Patients who had eye infection with occurrence of mucus and pus with the clinical diagnoses of bacterial conjunctivitis, keratoconjunctivitis, keratitis, blepharo-conjunctivitis, blepharitis, dacryo- cystitis and eye abscess were included in this study. The patients were of different ages, from 1 day to 15 years old. A total of 204 samples were collected from patients attending the Ophthalmology Teaching Hospital and Noor Al-Oyoon Hospital. Patients or alternatively their caretakers were signed informed consents. Purulent material from the surface of lower conjunctival sac and inner canthus of eye were aseptically collected by sterile saline pre-moistened swabs. In abscesses cases, abscesses were incised and the drained pus was obtained. This was done before the instillation of antimicrobial or steroidal eye-drops for treatment. Sample collection was done by ophthalmologists taking care of the participants. The samples were transferred to laboratory immediately in cold box for bacteriological examination.

Culture and identification

For each patient, a portion of the corneal scrape and the drained pus materias obtained were used for direct microscopy (Gram-stained smear) while the remaining material was inoculated directly onto the following media: blood agar, chocolate blood agar and MacConkey agar that support the growth of bacteria. Plates were incubated at temperature of 37°C. Aerobic atmospheric condition was maintained for the blood agar and the MacConkey agar while 10% carbon dioxide (CO_2) atmosphere was provided for the chocolate agar. All plates were initially examined for growth after 24 h and culture with no growth were re-incubated for further 48 h.

The bacteria isolated were identified by standard bacteriological test methods. Pure isolates of bacterial pathogens were preliminary characterized by colonial morphology and Gram-stain. Other biochemical procedures were used for full identification of Gram-positive and negative bacteria which included motility, indole, urease, oxidase, catalase and lactose fermentation tests. The pattern of utilization of X and V factors (Oxoid, Hampshire, UK) was employed for *Haemophilus* spp. characterization. Catalase and coagulase tests as well as haemolysis pattern on blood agar were used for identification of Gram-positive bacteria. Optochin sensitivity test was performed to identify *S. pneumoniae* and tributryin strips (Sigma Aldrich- Germany) were used for identification of *Moraxella catarrhalis*.

Table 1. Frequency of isolated bacteria in relation to gender and the various age groups.

Age (years)	Number	Gender		Percentage (%)
		Male	Female	
0-3	63	37	26	54
4-7	19	6	13	11
8-11	26	16	10	12
12-15	22	12	10	23
Total	130	71	59	100

Antimicrobial susceptibility testing

Isolates that were identified were tested for their susceptibility to a range of antibiotics using Kirby–Bauer disc diffusion test according to the clinical and laboratory standards institute (CLSI) guidelines. Antimicrobial susceptibility testing was performed for bacterial isolates by using the following antibiotics supplied by Oxoid Ltd.: amikacin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), co-trimoxazole (25 µg), ceftriaxone (30 µg) and amoxicillin (10 µg). Mueller-Hinton agars (oxoide) were used for the antibiotic sensitivity screen for non fastidious bacteria and 5% defibrinated blood was added along with Muller–Hinton agar for fastidious bacteria. Antibiotic discs added to Muller–Hinton plates were incubated at their respective optimal temperature and then the zones of inhibition diameters were measured. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines as sensitive, intermediate and resistant (CLSI, 2014).

Statistical analysis

Statistical analysis was performed using SPSS version 16 software. The associations of sociodemographic and clinical data to isolated pathogens were carried out by using the Pearson Chi-square test. P-value ≤ 0.05 was considered as statistically significant.

RESULTS

A total of 204 children with external bacterial eye infection were studied. Of all, 130 (63.7%) were males and 74 (36.3%) were females. Age ranges from 1 day to 15 years old with the mean age of the study subjects being 3.3 years (Table 1). The predominant age group was 0-3 years. Bacterial isolation in both sexes (P-value = 0.28) and various age groups (P-value = 0.58) did not show statistical significance. Out of 204 cultured eye

Table 2. Distribution of bacterial pathogens among clinical features of external ocular infection.

Bacterial isolates	Type of ocular infection							
	Conjunctivitis n=77	Kerato-conjunctivitis n=4	Keratitis n=27	Blepharo-conjunctivitis n=4	Abscesses n=4	Blepharitis n=10	Dacryo-cystitis n=4	Total
S. aureus	24	0		2	4	6	3	39(30%
S. pneumonia	9	0	17	0	0	4	1	31 (23%)
CoNS	3	0	2	0	0	0	0	5 (4%)
N. gonorrhoeae	6	4		0	0	0	0	10(7.7%)
P. aeruginosa	9	0	4	0	0	0	0	11(8.3%)
Klebsiella	2	0	2	0	0	0	0	4 (3.1%)
E. coli	2	0	0	0	0	0	0	2 (1.5%)
M.catarrhalis	2	0	0	2	0	0	0	4 (3.1%)
H. influenzae	20	0	2	0	0	0	0	22(17%)
Total	77 (59.2%)	4(3.1%)	27 (20.7%)	4 (3.1%)	4 (3.1%)	10 (7.7%)	4 (3.1%)	130(100%)

discharges, 130 (63.7%) bacterial isolates were identified.

Based on the clinical categorization, the predominant ocular bacterial infections were conjunctivitis (59.2%) followed by keratitis (20.7%), blepharitis (7.7%), keratoconjunctivitis (3.1%), dacryocystitis (3.1%), eye abscess (3.1%) and blepharoconjunctivitis (3.1%) (Table 2).

The orderly proportions of bacteria isolated regardless of their Gram staining were coagulase positive *S. aureus* 30.0% (39 of 130) followed by *S. pneumonia* 23.8% (31 of 130), *H. influenzae* 16.9% (22 of 130), *P. aeruginosa* 10% (13 of 130), *N. gonorrhoeae* 7.7% (10 of 130), CoNS (coagulase-negative *staphylococci*) 3.9% (5 of 130), *Klebsiella* 3.1% (4 of 130), *M. catarrhalis* 3.1% (4 of 130) and last of all *E. coli* 1.5% (2 of 130) (Table 3).

According to the categorization into Gram positive and negative, 57.7% of the bacterial isolates were Gram-positive (75 of 130) whereas, Gram negative bacteria comprised 42.3% of all isolates (55 of 130) (Table 2). Of the 75 isolated Gram-positive bacteria, the orderly proportions of the isolates were as follows: coagulase positive *S. aureus* 52.0% (39 of 75), *S. pneumonia* 41.3% (31 of 75) and CoNS 6.7% (5 of 75) (Table 2). Of the 55 isolated Gram-negative bacteria, the orderly proportions of the isolates were: *H. influenza* 40.0% (22 of 55), *P. aeruginosa* 23.6% (13 of 55) *N. gonorrhoeae* 18.2 % (10 of 55), *Klebsiella* 7.3% (4 of 55), *M.catarrhalis* 7.3% (4 of 55) and last of all *E. coli* 3.6% (2 of 55) (Table 2). The fractions of isolates from the different clinical entities studied were as follows: Conjunctivitis: *S. aureus* (31.1%), *H. influenza* (26%), *P. aeruginosa* (11.7%), *S.pneumoniae* (11.7%), *N. gonorrhoeae* (7.8%), CoNS (3.9%), *M. catarrhalis* (2.6%), *Klebsiella* spp. (2.6%) and *E. coli* (2.6%) (Table 2).

Kerato-conjunctivitis cases were totally caused by *N. gonorrhoeae (*100%) (Table 2). Keratitis cases were caused by *S. pneumonia* (63%), *P. aeruginosa,* (14.8%), CoNS (7.4%), *Klebsiella* spp. (7.4%) and *H. influenza* (7.4%) (Table 2). Blepharo-conjunctivitis cases were caused by S. *aureus* (50%) and *M. catarrhalis (*50%). Abscesses cases were totally caused by S*. aureus* (100%) (Table 2). Blepharitis cases were caused by S*. aureus* (60%) and *S. pneumonia (*40%) (Table 2). Dacryo-cystitis cases were caused by S*. aureus* (75%) and *S. pneumonia* (25%) (Table 2). Based on antibiotic susceptibility patterns, collectively, the Gram-positive cocci (*S. aureus,* CoNS and *S. pneumonia)* were highly sensitive to vancomycin (71 of 75; 95%), chloramphenicol and ciprofloxacin (68 of 75; 91%) for each followed by ceftriaxone (63 of 75; 84%). The majority of Gram-positives showed resistance against penicillin (54 of 75; 72%) as shown in Table 3. Individual antibiotic susceptibility patterns of Gram positive bacteria were as follows:

S. aureus showed high susceptibility to vancomycin (35 of 39; 90%), ciprofloxacin (33 of 39; 85%), chloramphenicol (32 of 39; 82%), amikacin (30 of 39; 77%), cotrimoxazole (30 of 39; 77%), gentamicin (29 of 39; 74%) and

Table 3. Antimicrobial susceptibility patterns of isolated bacteria.

Bacteria	No of isolates	s/r	P	AMP	TE	E	C	CN	CIP	CRO	AK	COT	VA
S. areus	39	S	0 (0)	14 (36)	11 (28)	28 (72)	32(82)	29 (74)	33(85)	29(74)	30(77)	30(77)	35(90)
		R	39 (100)	25 (64)	28 (72)	11(28)	7 (18)	10 (26)	6 (15)	10(26)	9(23)	9(23)	4(10)
CoNS	5	S	1 (20)	1(20)	2 (40)	4 (20)	5 (100)	1 (20)	5(100)	4(80)	3 (60)	2(40)	5(100)
		R	4 (80)	4 (80)	3 (60)	1(80)	0	4(80)	0	1(20)	2(40)	3(60)	0
S. pneumoniae	31	S	20 (64.5)	13(42)	15 (48)	26(84)	31(100)	22 (71)	30(97)	30(97)	22(71)	25(81)	31(100)
		R	11(35.5)	18(58)	16(52)	5(16)	0	9(29)	1(3)	1(3)	9(29)	14(19)	0
H. influenzae	22	S	NT	11(50)	18(82)	18(82)	19(86)	22(100)	20(91)	21(95)	18(81)	3(18)	NT
		R		11(50)	4(18)	4 (18)	3(14)	0	2(9)	1 (5)	4(19)	19(86)	
N. gonorrheae	10	S	7 (70)	4(40)	8(80)	9(90)	9(90)	0	10(100)	10(100)	10 (100)	2 (20)	NT
		R	3 (30)	7(60)	2(20)	1(10)	1(10)	10(100)	0	0	0	8(80)	
P. aeruginosa	13	S	-	0	2 (15)	NT	1(9)	0	6(46)	8(62)	13(100)	1(8)	NT
		R	-	13(100)	11(85)	NT	12(91)	13(100)	7(54)	5(38)	0	12(92)	
Klebsiella spp.	4	S	-	1(25)	0	-	1(25)	2(50)	2(50)	3(75)	4(100)	1(25)	NT
		R	-	3(75)	4(100)	-	3(75)	2(50)	2(50)	1(25)	0	3(75)	
M. catarrhalis	4	S	0	0	3(75)	NT	4(100)	4(100)	4(100)	4(100)	4(100)	1(25)	NT
		R	4	4	1(25)	NT	0	0	0	0	0	3(75)	
E.coli	2	S	-	0	1(50)	NT	0	1(50)	1(50)	2(100)	2(100)		NT
		R	-	2(100)	1(50)	NT	2(100)	1(50)	1(50)	0	0		

CoNS=Coagulase negative staphylococcus, S=sensitive, R= resistance, P=penicillin AMP=Ampicillin, TE=tetracyclin, E=erythromycin, C= chloramphenicol, CN=gentamicin, CIP=ciprofloxacin, CRO=cefrtiaxone, AK= amikacin, COT=cotrimoxazole, VA= vancomycin.

erythromycin (28 of 39; 72%). On the other hand, they were totally resistant to pencillin (39 of 39; 100.0%) and resistant to lesser extents to tetracycline (28 of 39; 72%) and ampicillin (25 of 39; 64%) (Table 3). *S. pneumoniae* showed complete susceptibility to vancomycin and chloramphenicol (31 of 31; 100%) for each and lesser susceptibility to ceftriaxone and ciprofloxacin (30 of 31; 97%) for each, erythromycin (26 of 31; 84%) and cotrimoxazole (25 of 31; 81%.) conversely, more than half of *S. pneumoniae* showed resistance against ampicillin (18 of 31; 58%) and tetracycline (16 of 31; 52%) (Table 3). CoNS were totally susceptible to vancomycin, ciprofloxacin and chloramphenicol (5 of 5; 100%) for each. The majority of bacteria isolates were resistant to penicillin, ampicillin and gentamicin (4 of 5; 80%) for each (Table 3). For Gram-negative organisms (*H. influenzae*, *P. aeruginosa*, *N. gonorrhoeae*, *Klebsiella*, *M. catarrhalis* and *E. coli)* as a group, the highest susceptibility was to amikacin (92.7%; 51 of 55), followed by ceftriaxone (87.3%; 48 of 55) and ciprofloxacin (78.2%; 43 of 55). Major resistance was to cotrimoxazole (45 of 55; 82%) and ampicillin (40 of 55; 73%) (Table 3). Individual antibiotic susceptibility pattern of Gram negative bacteria were as follows: *H. influenzae* were found to be sensitive to gentamicin (22 of 22; 100%) followed by cefrtiaxone (21 of 22; 95%), ciprofloxacin (20 of 22; 91%), chloramphenicol (19 of 22; 86%) and amikacin (18 of 22;81%), whereas the highest resistance was to cotrimoxazole (19 of 22; 86%) (Table 3).

P. aeruginosa showed complete sensitivity to amikacin (13 of 13; 100.0%) and less sensitivity to cefrtiaxone (8 of13; 62.0%). However, they displayed complete resistance towards gentamicin and ampicillin (13 of 13; 100.0%) for each and

high resistance to cotrimexazole and chloramphenicol (12 of 13; 92.0%) for each followed by tetracycline (11 of 13; 85.0%). *N. gonorrhoeae* were fully susceptible to amikacin, ceftriaxone, ciprofloxacin and gentamicin (10 of 10; 100%) for each. Conversely, they showed extreme resistance to gentamicin (10 of 10; 100%) followed by cotrimoxazole (8 of 10; 80%) and ampicillin (7 of 10; 60%) (Table 3). *Klebsiella* species showed full susceptibility to amikacin (4 of 4; 100%), total resistance to tetracycline (4 of 4; 100%) and high resistance to ampicillin, cotrimoxazole and chloramphenicol (3 of 4; 75%) for each.

M. catarrhalis were fully susceptible to amikacin, ceftriaxone, ciprofloxacin, chloramphenicol and gentamicin (4 of 4; 100%) for each and fully resistant to penicillin and ampicillin (4 of 4; 100%) for each.

E. coli were completely susceptible to amikacin and ceftriaxone (2 of 2; 100%). 50% strains of *E. coli* species were sensitive to ciprofloxacin, gentamicin and tetracycline, whereas the isolates showed full resistance to both ampicillin and chloramphenicol (2 of 2; 100%).

DISCUSSION

The eye and its associated structures are uniquely predisposed to infections by various organisms, mainly, bacteria, viruses and fungi and rarely parasites. The results of the current study are showing significant similarities as compared to other analogous ones, nevertheless when it comes to certain particular aspects, striking differences has been noted between the present findings and their equivalent ones in other surveys.

In this study, the overall prevalence of bacterial eye infections was 63.7% where as 36.3% of clinical samples did not show bacterial growth. Similar studies conducted in Ethiopia concluded that the prevalence was 54.2, 60.8, 59.4 and 60.8% (Anagaw et al., 2011; Muluye et al., 2014; Assefa et al., 2015; Shiferaw et al., 2015). Parallel works in India concluded the prevalence was 58.8 and 61% (Bharathi et al., 2010; Ramesh et al., 2010). The cause of absence of bacterial growth in clinically diagnosed cases might be bacterial causes not identified by the conventional laboratory parameters or non-bacterial causes like viruses and fungi or non infective causes like eye allergies.

Almost half of the bacterial isolates (48.5%) were from patients in the age group of less than three years of life. Susceptibility to infection is increased in babies due to low immunity at such ages (Niewiesk, 2014). In addition to this, the air facilitates the transfer of bacteria to hospital delivery rooms especially when opening the doors and windows (Al-Oqaili., 2004).

Gram positive bacteria were the dominant isolate (57.7%) in the current study. This is supported by other studies conducted in Ethiopia and Nigeria (Bharathi et al., 2010; Shiferaw et al., 2015). Among the Gram positive bacteria, *S. aureus* was the most common pathogen with an overall prevalence of 30% equivalent to 52% of Gram positive bacteria. Previous reports showed that *S. aureus* was the most predominant isolated pathogen from ocular infections (Chaudhry et al., 2005a; Ubani, 2009; Bharathi et al., 2010; Ramesh et al., 2010; Anagaw et al., 2011; Tesfaye et al., 2013; Musa et al., 2014). These findings reflect the known high virulence of these bacteria. Other studies showed that that CoNS was the most frequent bacteria involved in eye infections (Anagaw et al., 2011; Muluye et al., 2014; Assefa et al., 2015). This may be due variations in climatic, geographical and ethnic parameters.

Gram negative bacteria were less dominant (43.3%) with *H. influenza* heading the list with an overall prevalence of 17% of all isolates equivalent to 40% of Gram negative isolates. On the other hand, *M. catarrhalis* had a lesser frequency (3.1%) of all isolates. This finding is in agreement with a previous study (Tesfaye et al., 2013). However, in other works, the major isolate was *M. catarrhalis* (Bharathi et al., 2010), *E. coli* (Assefa et al., 2015), *Proteus* species (Musa et al., 2014) and *P. aeruginosa* (Tesfaye et al., 2013).

This study showed limited isolates of enteric bacteria (5%) which is comparable to the finding of another study (Tesfaye et al., 2013) and contrary to those of other ones (Esenwah, 2005; Anagaw et al., 2011). This can be ascribed to factors related to the communities studied and their surrounding conditions plus biological disparities of the isolates. The study showed that the most common bacterial ocular infections is conjunctivitis (59.2%), the second most frequent one is keratitis (20.7%) followed by blepharitis (7.7%) whereas keratoconjunctivitis, dacryocystitis, blepharoconjunctivitis and eye abscesses had the same frequency (3.1%).

In the present work, many bacteria were isolated from patients with conjunctivitis. These are in the order: *S. aureus* (31.1%), *H. influenza,* (26%), *P. aeruginosa* (11.7%), *S. pneumoniae* (11.7%), *N. gonorrhoeae* (7.8%), CoNS (3.9%), *M. catarrhalis* (2.6%), *Klebsiella* spp. (2.6%) and *E. coli* (2.6%). Comparable studies reported *S. aureus* as the predominant bacteria in conjunctivitis (Bharathi et al., 2010; Ramesh et al., 2010).

S. pneumoniae was found to be the main isolate in cases of microbial keratitis (63%) followed by *P. aeruginosa,* (14.8%), CoNS (7.4%), *Klebsiella* spp. (7.4%) and *H. influenzae* (7.4%). This finding is in agreement with those of similar studies conducted in Ethiopia and India (Alemayehu, 2004; Bharathi et al., 2010). One study in India reported *P. aeruginosa* and *S. pneumoniae* as the predominant isolates of microbial keratitis with equal frequency (Geethakumari et al., 2011). In contrast, other studies reported P*. aeruginosa* as the major isolate (Bharathi et al., 2010; Tesfaye et al., 2013). Another study concluded that *S. aureus* are the most common bacterial pathogens isolated in keratitis (Kaliamurthy et al., 2013). This may be due to inter-

population variations and environmental dissimilarities in different countries (Janumala et al., 2012). The most common infection of lacrimal apparatus is dacryocystitis (Ramesh et al., 2010).

Obstruction of the nasolacrimal duct provides a good environment for bacterial proliferation leading to secondary bacterial infection (Ramesh et al., 2010). In this study, the prevalence of *S. aureus* was the highest among dacrocystitis isolates followed by *S. pneumoniae*. This is consistent with the findings of other surveys (Chaudhry et al., 2005b; Assefa et al., 2015) and is discordant with other works which reported *S. pneumoniae* as the most prevalent isolate (Ramesh et al., 2010; Kebede et al., 2010).

In this study, *S. aureus* was the sole isolated bacteria in all cases of orbital cellulitis with eyelid abscess. This finding is compatible with previous studies that reported *S. aureus* as the predominant pathogen involved in this type of ocular infections (Blomquist, 2006; Akçay et al., 2014). From the abovementioned findings and comparative analysis, different organisms are implicated in the etiology of infections targeting this disease-prone organ in the population under study. Some of these organisms are part of normal flora and some are not.

Despite the fact that *Staphylococci* and *Streptococci* along with other bacteria like *Haemophilus*, *Moraxella* and some *Neisseria* spp. are part of the normal flora of the conjunctiva; under certain circumstances they become involved in ophthalmic infections (Bharathi et al., 2010). Non-pathogenic *Neisseria* spp. that are normal commensals of mucosal surfaces include *N. lactamica*, *N. sicca* and others (Liu et al., 2015).

The principle routes of acquisition of the pathogens are airborne droplets, eye contact with contaminated hands and spread from nearby body sites (Ramesh et al., 2010). A variety of virulence and predisposing factors are involved in these infections.

S. aureus which heads the lists of pathogenic bacteria in the current study possess cell surface factors like antiphagocytic capsule and secreted virulence factors like hemolysins and leukocidin (Costa et al., 2013). *S. pneumoniae* which comes in the second place is frequently found in the lacrimal apparatus and conjunctiva as normal flora. Minor corneal epithelial disruption predisposes to its invasion and consequently corneal infection (Bharathi et al., 2010). High virulence of *H. infleuenzae* which occupies the third position can be explained by features like capsule, adhesions molecules and pilli (Kostyanev and Sechanova, 2012). Concerning the less prevalent organisms in the current work, *Pseudomonas* possess factors like glycocalyx and pili for adherence while the biofilms which coat them facilitate their attachment to their targets (Al-Mujaini et al., 2009). They also cause corneal stroma melting due to enzymatic effects (Marquart et al., 2013). *N. gonorrhoeae* virulence factors include pili, porin and Opa proteins (van Vliet et al., 2009). Low immunity and medical devices use predispose to CoNS infections (Becker et al., 2014). *Moraxella* spp. use adhesions and can evade complement system (Perez Vidakovics and Riesbeck, 2009). *Klebsiella* have prominent antiphagocytic capsule and *magA* (Hunt et al., 2011). Vulnerability to *E. coli* induced conjunctivitis is related to young ages (Wiwanitkit, 2011) especially neonates in healthcare institutes (Goel et al., 2016).

Gram-positive cocci were highly susceptible to vancomycin (95%), chloramphenicol and ciprofloxacin (91%) and ceftriaxone (84%). A similar study conducted in India revealed that vancomycin and chloramphenicol had the highest efficacy against Gram positive isolates (Ramesh et al., 2010; Bharathi et al., 2010). These findings contrast with those obtained from studies carried out in India and Ethiopia which reported higher susceptibility to ciprofloxacin as compared to vancomycin (Bharathi et al., 2010; Tesfaye et al., 2013). However, one study conducted in Iran reported low coverage of vancomycin against *S. aureus* (Khosravi et al., 2007). The persistent conclusion of vancomycin as an antibiotic with high effectiveness against ocular Gram-positive isolates might be attributed to the fact that vancomycin inhibits early stages in cell wall mucopeptide synthesis.

In this study, all *S. aureus* strains were resistant to penicillin. This is very similar to previous reports from the studies performed among Ethiopian ocular infections (Bharathi et al., 2010; Anagaw et al., 2011). This can be explained by the well-known fact that most of *S. aureus* strains produce penicillinase and alternative penicillin binding protein (PBP-2A) rendering them resistant to most beta lactam antibiotics.

Gram negative bacteria were highly susceptible to amikacin (92.7%) followed by ceftriaxone (87.3%) and lastly ciprofloxacin (78.2%). This observation is consistent with those obtained from studies conducted in India, Ethiopia and Libya (Bharathi et al., 2010; Tesfaye et al., 2013; Musa et al., 2014). Yet, it is contradictive to the findings obtained from a recent study in Ethiopia which reported high susceptibility of Gram negative isolates to gentamicin along with resistance to ceftriaxone and ciprofloxacin (Anagaw et al., 2011).

Conclusion and recommendations

Bacterial external ocular infections are of considerable prevalence among Sudanese pediatrics population. They are the cause of no less than two thirds of all cases. Gram positive bacteria constitute more than half of the isolates with *Staphylococcus* species being the most predominant ones. However, Gram negative bacteria have comparatively significant contribution to this category of diseases. Different antimicrobial susceptibility patterns were identified. Consequently, identification of potential pathogenic bacteria implicated in these infections through culture and biochemical tests

techniques as well as recognition of drug susceptibility pattern must be carried out as routine diagnostic laboratory tests for proper management. The etiological agents responsible for a considerable fraction of clinically diagnosed cases were not identified by conventional methods. Accordingly, workup of other causes like bacteria not recognized by traditional methods is required including sophisticated techniques such as molecular modalities. Non-bacterial infective organisms and non-infective agents should also be investigated.

Conflicts of interest

The authors declare that there is no conflict of interest.

REFERENCES

Akçay E, Can GD, Çağıl N (2014). Preseptal and orbital cellulitis. J Microbiol. Infect. Dis. 4(3):123-127.

Akpek EK , Gottsch JD (2003). Immune defense at the ocular surface. Eye 17:949-956.

Alemayehu N (2004). Pattern of Microbial Agents of External Ocular Infections in Federal Police Hospital and Minilik II Memorial Hospital. Addis Ababa, Ethiopia, 69P. (MSc Thesis. Faculty of Medicine, Addis Ababa University).

Al-Mujaini A, Al-Kharusi N, Thakral A, Wali UK (2009). Bacterial Keratitis: Perspective on Epidemiology, Clinico-Pathogenesis, Diagnosis and Treatment. Sultan Qaboos Univ. Med. J. 9:184-195.

Al-Oqaili N (2004). Study of microbial eye infection of newborn babies in Diwaniya city. College of education. Al-Qadisiya University. MSc Thesis. Pp. 44-54.

American Academy of Ophthalmology Cornea/External Disease Panel (2011). Preferred Practice Patterns® Guidelines. Conjunctivitis – Limited Revision. San Francisco: American Academy of Ophthalmology; 2011. Available at: www.aao.org/ppp (accessed 10/28/11).

Anagaw B, Biadglegne F, Belyhun Y, Mulu A (2011). Bacteriology of ocular infections and antibiotic susceptibility pattern in Gondar University Hospital, North west Ethiopia. Ethiop. Med. J. 49(2):117-123.

Assefa Y, Moges F, Endris M, Zereay B, Amare B, Bekele D, Tesfaye S, Mulu A, Belyhun Y (2015). Bacteriological profile and drug susceptibility patterns in dacryocystitis patients attending Gondar University Teaching Hospital, Northwest Ethiopia. BMC Ophthalmol. 15(1):1.

Bartlett JD, Jaanus S (2008). Clinical Ocular Pharmacology. St. Louis, MO: Butterworth Heinemann Elsevier.

Becker K, Heilmann C, Peters G (2014). Coagulase-Negative *Staphylococci*. Clin. Microbiol. Rev. 27:870-926.

Bertino JS (2009). Impact of antibiotic resistance in the management of ocular infections: the role of current and future antibiotics. Clin. Ophthalmol. 3:507-521.

Bharathi J, Ramakrishnan R, Shivakumar C, Meenakshi R, Lionalraj D (2010). Etiology and antibacterial susceptibility pattern of community-acquired bacterial ocular infections in a tertiary eye care hospital in south India. Indian J. Ophthalmol. 58:497-507.

Blomquist PH (2006). Methicillin-resistant *Staphylococcus aureus* infections of the eye and orbit (An American Ophthalmological Society Thesis). Trans. Am. Ophthalmol. Soc. 104:322-345.

Bolaños-Jiménez R, Navas A, López-Lizárraga EP, de Ribot FM, Peña A, Graue-Hernández EO, Garfias Y (2015). Ocular Surface as Barrier of Innate Immunity. Open Ophthalmol. J. 9:49-55.

Briscoe D, Rubowitz A, Assia E (2005). Changing bacterial isolates and antibiotic sensitivities of purulent dacryocystitis. Orbit 24(2):95-98.

Brissette A, Schweitzer K, Arthur B (2011). Answer: Can you identify this condition? Can. Fam. Physician 57(1):55.

Cavuoto K, Zutshi D, Karp CL, Miller D, Feuer W (2008). Update on bacterial conjunctivitis in South Florida. Ophthalmology 115(1):51-56.

Chaudhry IA, Al-Rashaed W, Shamsi FA (2005a). Microbial profile and prevalence of acute dacryocystitis in adult patients with chronic dacryocystitis. Saudi J. Ophthalmol. 19:93-8.

Chaudhry IA, Shamsi FA, Al-Rashed W (2005b). Bacteriology of chronic dacryocystitis in a tertiary eye care center. Ophthal. Plast. Reconstr. Surg. 21:207-210.

CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement (2014). CLSI document M100-S24. Wayne, PA: Clinical and Laboratory Standards Institute.

Costa AR, Batistão DWF, Ribas RM, Sousa AM, Pereira O, Botelho CM (2013). *Staphylococcus aureus* virulence factors and disease in microbial pathogens and strategies for combating them: Science, Technology and Education, ed. Méndez-Vilas A., editor. (Badajoz: Formatex Research Center. pp. 702–710.

Esenwah E (2005). Isolation and Identification of the Microorganisms most Prevalent in External Eye Infections as seen in an Eye Clinic in Owerri. J. Niger. Optom. Assoc.12:6-9.

Geethakumari PV, Remya R, Girijadevi MS, Reena A (2011). Bacterial keratitis and fungal keratitis in South Kerala: a comparative study. Kerla J. Ophthalmol. 23:43-46.

Goel K, Randhawa VS, Saili A, Khare S, Kumar A, Dutta R, Goel G (2016). Incidence, Etiology and Risk Factors Associated with Neonatal Healthcare-Associated Conjunctivitis: A Prospective Study from a Tertiary Care Hospital in India. J. Trop. Pediatr. 62:10-18.

Gonzalez MO, Durairaj VD (2010). Understanding Pediatric Bacterial Preseptal and Orbital Cellulitis. Middle East Afr. J Ophthalmol. 17:134-137.

Hovding G (2008). Acute bacterial conjunctivitis. Acta Ophthalmol. 86(1):5-17.

Hunt JJ, Wang J T, Callegan MC (2011). Contribution of Mucoviscosity-Associated Gene A (magA) to Virulence in Experimental *Klebsiella pneumoniae* Endophthalmitis. Invest. Ophthalmol. Vis. Sci. 52:6860-6866.

Janumala H, Sehgal PK, Mandal AB (2012). Bacterial Keratitis - Causes, Symptoms and Treatment, Keratitis, Dr. Muthiah Srinivasan (Ed.), ISBN: 978-953-51-0568-8, InTech, DOI: 10.5772/34599. Available from: http://www.intechopen.com/books/keratitis/bacterial-keratitis-causes-symptoms-and-treatment.

Kaliamurthy J, Kalavathy CM, Parmar P, Nelson Jesudasan CA, Thomas PA (2013). Spectrum of Bacterial Keratitis at a Tertiary Eye Care Centre in India. Biomed. Res Int. Volume 2013 (2013), Article ID 181564, 8 pages.

Kebede A, Adamu Y, Bejiga A (2010). Bacteriological study of dacryocystitis among patients attending in Menelik II Hospital, Addis Ababa, Ethiopia. Ethiop. Med. J. 48(1):29-33.

Khosravi AD, Mehdinejad M, Heidari M (2007). Bacteriologic Findings in Patients with Ocular Infection and Antibiotic Susceptibility Patterns of Isolated Pathogens. Singapore. Med. J. 48(8):741-743.

Kostyanev TS, Sechanova LP (2012). Virulence factors and mechanisms of antibiotic resistance of haemophilus influenzae. Folia Med. 54:19-23.

Kubal A, Garibaldi D (2008). Dacryoadenitis caused by methicillin resistant *Staphylococcus aureus.* Ophthal. Plast. Reconstr. Surg. 24(1):50-61.

Linden SK, Sutton P, Karlsson N G, Korolik V, McGuckin MA (2008). Mucins in the mucosal barrier to infection. Mucosal Immunol. 1:183-197.

Liu G, Tang CM, Exley RM (2015). Non-pathogenic *Neisseria*: members of an abundant, multi-habitat, diverse genus. Microbiology 161:1297-1312.

Marquart ME, O'Callaghan RJ, Marquart ME, O'Callaghan RJ (2013). Infectious Keratitis: Secreted Bacterial Proteins That Mediate Corneal Damage, Infectious Keratitis: Secreted Bacterial Proteins That Mediate Corneal Damage. J. Ophthalmol. Volume 2013, Article ID 369094, 9 pages.

Muluye D, Wondimeneh Y, Moges F, Nega T, Ferede G (2014). Types and drug susceptibility patterns of bacterial isolates from eye discharge samples at Gondar University Hospital, Northwest Ethiopia. BMC Res. Notes 7(1):1.

Musa AA, Nazeerullah R, Sarite SR (2014). Bacterial profile and

antimicrobial susceptibility pattern of anterior blepharitis in Misurataregion, Libya. Dent. Med. Res. 2(1):8-13.
Niewiesk S (2014). Maternal Antibodies: Clinical Significance, Mechanism of Interference with Immune Responses, and Possible Vaccination Strategies. Front. Immunol. 5:446.
Perez Vidakovics ML, Riesbeck KC (2009). Virulence mechanisms of *Moraxella* in the pathogenesis of infection. Curr. Opin. Infect. Dis. 22:279-285.
Rahimi F, Hashemian MN, Khosravi A, Moradi G, Bamdad S (2015). Bacterial keratitis in a tertiary eye centre in Iran: A retrospective study. Middle East Afr. J. Ophthalmol. 2(2):238-244.
Ramesh S, Ramakrishnan R, Bharathi J, Amuthan M, Viswanathan S (2010). Prevalence of bacterial pathogens causing ocular infections in South India. Ind. J. Pathol. Microbiol. 53:281-286.
Rutar T, Zwick OM, Cockerham KP, Horton JC (2005). Bilateral blindness from orbital cellulitis caused by community-acquired methicillinresistant *Staphylococcus aureus.* Am J. Ophthalmol. 140(4):740-742.
Sharma S (2011). Antibiotics and Resistance in Ocular Infections. Indian J. Med. Microbiol. 29:218-222.
Shiferaw B, Gelaw B, Assefa A, Assefa Y, Addis Z (2015). Bacterial isolates and their antimicrobial susceptibility pattern among patients with external ocular infections at Borumeda hospital, Northeast Ethiope. BMC Ophthalmol. 15:103.
Stratton M, Capitena C, Christensen L, Paciuc-Beja M, Stratton M, Capitena C , Christensen L, Paciuc-Beja M (2015). *Escherichia coli* Eyelid Abscess in a Patient with Alcoholic Cirrhosis, Case Reports in Ophthalmological Medicine. Case Rep. Ophthalmol. Med. 2015:9.
Tesfaye T, Beyene G, Gelaw Y, Bekele S , Saravanan M (2013). Bacterial Profile and Antimicrobial Susceptibility Pattern of External Ocular Infections in Jimma University Specialized Hospital, Southwest Ethiopia. Am. J. Infect. Dis. Microbiol. 1:13-20
Ubani UA (2009). Bacteriology of external Ocular Infections in Aba, South Eastern Nigeria. Clin. Exp. Optom. 92(6):482-489.
Venkataraman M, Krishnagopal S, Sivaraman U, Ramachandran RA (2015). Spectrum of Conjunctival Bacterial Flora in Non-Insulin Dependent Diabetics, India. Int. J. Sci. Stud. 3:133-136.
van Vliet SJ, Steeghs L, Bruijns SC, Vaezirad MM, Blok CS, Busto JA, Deken M, van Putten JP, van Kooyk Y (2009). Variation of *Neisseria gonorrhoeae* Lipooligosaccharide Directs Dendritic Cell–Induced T Helper Responses. PLOS Pathog. 5(10):e1000625.
Wiwanitkit V (2011). *E. Coli* Outbreak and Conjunctivitis. Iran J. Ophthalmol. 23:72-72.

academicJournals

Vol. 10(40), pp. 1703-1710, 28 October, 2016
DOI: 10.5897/AJMR2016.8267
Article Number: 88E7DB861336
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological and molecular characterization of environmental mycobacterium strains isolated from the Buruli ulcer endemic and non-endemic zones in Côte d'Ivoire

Sabine N. Vakou[1,2]*, Julien Coulibaly-Kalpy[1,2], Solange E. Kakou-Ngazoa[1], N`guetta Aka[1], N`golo D. Coulibaly[1], Mireille Dosso[1] and Allico J. Djaman[1,2]

[1]Pasteur Institute of Ivory Coast, 01 BP 490 Abidjan, Côte d'Ivoire.
[2]University of Felix Houphouët Boigny, Ivory Coast, Côte d'Ivoire

Received 12 August 2016, Accepted 27 September, 2016

***Mycobacterium ulcerans (MU)*, the causative agent of Buruli ulcer (BU), skin disease, is considered to be an environmental pathogen. The pathogenic virulence of *MU* is being linked to the expression of toxin called Mycolactone. Genetic analyses have shown the high diversity with variable number tandem repeats (VNTR) and mycobacterial interspersed repetitive units (MIRU) in *M. ulcerans* and in mycolactone producing Mycobacteria (MPMs). The purpose of this study is the molecular characterization of potentially pathogenic environmental mycobacteria strain, apart from the *M. ulcerans,* from aquatic environments in Côte d'Ivoire. A total of 473 samples were collected comprising of 251 water and 222 sediment based on sampling sites. The sediments were the most contaminated by mycobacteria with 60% as against 43.3% in water samples from the hyper endemic areas. In hypo-endemic areas, water was the most contaminated with 53.57% against 43.24% in sediment. Microscopy by Ziehl-Neelsen-staining, and PCR diagnostics using IS2404 and KR were performed on strains. 20% fast growing isolated mycobacteria species including *Mycobacterium mucogenicum*, *Mycobacterium peregrinum* and *Mycobacterium* sp. was found carrying the IS2404 gene previously found in *M. ulcerans.* 9.23% of strains carry the ketoreductase (KR) genes, one of the synthesis of mycolactone enzymes. In terms of genetic analysis using the MIRU/VNTR, the MIRU 1 was the most amplified sequence, and LOCUS 6 less amplified; no known profile have been identified in this study. This study is the first step taken in order to understand different skin infections encountered in Côte d'Ivoire.**

Key words: Mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR; ketoreductase, LOCUS 6, IS2404, *Mycobacteria*, Buruli ulcer.

INTRODUCTION

Mycobacteria are germs that cause lung infections, skin or lymph (Griffith et al., 2007). They are found in the environment like in the soil, water, aerosols, plants, aquatic animals (Winthrop et al., 2002; Sniezek et al.,

*Corresponding author. E-mail: vsalger@yahoo.fr. Tel: +22508837644.

2003; Marsollier et al., 2002, 2004). This Acid-Alcohol Bacillus Resistant (AFB) are not mandatory pathogens and for several years, the scientific community have not really shown interest in their study, but rather devoted to the study of TB epidemic. They were considered as saprophytic bacteria and their pathogenic potential are not recognized (Ziza and Desplaces, 2006). However, some mycobacteria such as *Mycobacterium liflandii* and *Mycobacterium fortuitum* produce toxins causing skin infections (Williamson et al., 2008; Kakou-Ngazoa et al., 2015). This is an extremely polymorphic bacterial genus that includes fast-growing species (less than 7 days), and slow growing species (7-60 days) and non-culturable outside animals species, *Mycobacterium leprae*, leprosy causing agent (Euzéby, 2010). Mycobacteria are divided into two major groups, they are complex tuberculosis and non-tuberculosis mycobacteria (NTM) also called environmental mycobacteria (mycobacteria of leprosy and atypical mycobacteria) (Inderlied et al., 1993; Caruso et al., 2009; del Rio Camacho et al., 2010). Although, molecular biology has helped to discover the existence of diversity in environmental mycobacteria (Domenech et al., 1994; Menendez et al., 2002; Williamson et al., 2008; Kakou-Ngazoa et al., 2015), culturing of species however, remains the most efficient means of knowing their physiology and their antibiotic sensitivity (Kubica et al., 1964; Trujillo et al., 2004). The most important ones are *Mycobacterium avium*, *Mycobacterium intracellulare, Mycobacterium kansasii, Mycobacterium xenopi* and *Mycobacterium abscessus* (Falkinham, 1996; Dailloux et al., 2010). The complex *M. avium, M. kansasii, M. chelonae* and *M. xenopi* are responsible for the majority of infections in the developed countries (Horsburgh, 1996), while *M. ulcerans* is responsible for ulcerative diseases in tropical and subtropical countries (Pedley et al., 2004). The distribution of commonly isolated species is in constant change in most countries studied and new species emerge (Martin-Casabona et al., 2004). They are part of the group of non-pigmented genes atypical mycobacteria such as *M. abscessus, M. chelonae, M. fortuitum* and *M. smegmatis* (Brown and Wallace, 1992).

Buruli ulcer, debilitating disease, is a serious public health problem. Almost all regions in Côte d'Ivoire are affected. The mode of transmission and environmental sources are unknown. To be able to understand the level of prevalence of skin infections in Côte d'Ivoire, it is important to identify species other than *M. ulcerans* that are involved. Given their large numbers, this study was undertaken with the aim to achieve the molecular characterization of potentially pathogenic environmental mycobacteria strains others than *M. ulcerans* from different aquatic environments in Côte d'Ivoire.

MATERIALS AND METHODS

Sites and scope of the study

The study was conducted at different sites considered to be Buruli ulcer endemic zones (Adiopodoumé, Tiassalé, Adzopé) and non-endemic zones (Agboville, Bouaké, Aghien) according to the national program against Buruli ulcer in Côte d'Ivoire. However, all biological part of this work was carried out at the Pastor Institute of Côte d'Ivoire.

Biological material

The biological material consisted of water and sediments sample from different studied environments.

Procedure

Samples were collected monthly, from June 2014 to June 2015. A total of 22 sampling stations were selected, 11 from the Lagoon of Aghien, 3 from Adzopé water retention. 2 stations were selected from Sokrogbo sites, Bodo and Adiopodoumé, respectively, 1 station each was selected, at the entrance of Agboville and Loka in Bouake water retention (Figure 1). The real prevalence of MU in the sampling site is unknown. But according the National reference Center of Buruli Ulcer (unpublished document), the rate of Buruli Ulcer confirmation in the region of the sampling site were: 62% Agboville; 56.36% Bouake, 30.77% Aghien and Adzopé.

Collection of water and sediment samples

The sediment samples were collected using dump Eckmann at big water points and a sampler at the banks (Schiavone and Coquery, 2011). A 5 L capacity bucket spout allowed drawing water at the bank of water points and a hydrological bottle 1.5 L capacity for drawing water far away from the banks. The sediments samples were put in sterile plastic bags and the water samples in sterile glass bottles of 1 L capacity. The samples were then kept refrigerated at 4°C during transportation, protected from light and taken to the laboratory within 24 h of sampling.

Culturing

In the laboratory, 500 g of each sediment and 100 mL of water sample was collected. In a Falcon tube 50 mL, 10 g of sediment were mixed with 40 mL of sterile distilled water following the method described by Kankya et al. (2011), slightly modified. After mixture of sediment and distilled sterile water, the supernatant was recovered in a new Falcon tube. Decontamination of water samples and recovered supernatant was carried out with cetylpiridium chloride (CPC) (Stinear et al., 2004), followed by neutralization with phosphate buffer.

The different culture medium: Loweinstein Jensen (LJ), Mac Conkey without purple crystal, ordinary agar and Middlebrook 7H10 agar was used for seeding. The samples in LJ and Middlebrook 7H10 agar were seeded in duplicate. One lot of each of them was packed in an aluminum foil for photoinduction test. The incubations were made at 23 and 37°C in the ovens. Daily observation were made until colonies were obtained.

An optical microscope (*Zeiss*®) was used for the observation of Acid-Alcohol Bacillus Resistant after Ziehl-Neelsen coloration (Barksdale, Kim. 1977). Classification of species was made according to the method described by Runyon and collaborators (1959). Biochemical identification of mycobacteria was done according to the method described by Metchock (1995). Briefly, for biochemical identification, the colonies obtained after culture was observed microscopically. The Acid-Alcohol Bacillus Resistant were cultured in the presence and absence of light on LJ medium for the test of photo-induction.

They were then seeded on ordinaire Agar at different temperatures (37, 42, 45 and 52°C), on LJ medium with 5% NaCl with the manitol test, the test in presence of sodium citrate and test in the presence of ferric ammonium.

Table 1. List of primers and detectors used.

Detector	Primers	Sequences (5'-3')	References
IS6110	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	Wilton and Cousin (1992)
	MYCGEN-R	TGCACACAGGCCACAAGGGA	
IS2404	IS2404 F	ATTGGTGCCGATCGAGTTG	Ross et al. (1997)
	IS2404 R	TCGCTTTGGCGCGTAAA	
	IS2404-probe	6 FAM-CACCACGCAGCATTCTTGCCGT-TAMRA	
KR	KR F	TCACGGCCTGCGATATCA	Fyfe et al. (2007)
	KR R	TTGTGTGGGCACTGAATTGAC	
	KR-probe	6 FAM-ACCCCGAAGCACTG-TAMRA	
MIRU1	MIRU1 F	GCTGGTTCATGCGTGGAAG	Stragier et al. (2005), Ablordey et al. (2005) and Hilty et al. (2006)
	MIRU1 R	GCCCTCGGGAATGTGGTT	
ST1	ST1 F	CTGAGGGGATTTCACGACCAG	
	ST1 R	CGCCACCCGCGGACACAGTCG	
VNTR-19	Locus 19F	CCGACGGATGAATCTGTAGGT	
	Locus 19R	TGGCGACGATCGAGTCTC	
VNTR-6	Locus 6 F	GACCGTCATGTCGTTCGATCCTAGT	
	Locus 6 R	GACATCGAAGAGGTGTGCCGTCT	

Molecular analysis of strains of mycobacteria

The DNA extraction was performed according to the method described by Ausubel et al. (1987). Molecular characterization was carried out using two types of PCR: Conventional PCR for the search of sequences IS6110 and MIRU-VNTR (Miru 1, Locus 6 VNTR19; ST1) and RT-PCR for the IS2404 sequences and ketoreductase (Kr). These analyses were done with all isolated colonies of Acid Alcohol Bacillus Resistant after culture. IS6110 and IS2404 were chosen to identify mycobacteria, MIRU-VNTR for typing the mycobacteria and the Kr sequence for the search of virulence factor.

Conventional PCR

The amplification of sequences IS6110 and Miru VNTR was made in the presence of specific primers for each sequence (Table 1). Each DNA extract (5 µL) was placed in the presence of 10X buffer, magnesium chloride (25 mM), DNTP 10 µM, copies of primers specific to each sequence (IS6110 and Miru VNTR), of 0.2 µl of DNA Taq polymerase (Hot Start Taq) in a final volume of 50 µL. The reaction mixture was incubated in a thermocycler of the type Gene Amp 9700 (Applied Biosystem®), according to the following schedule:IS6110 (94°C for 5 min; 94°C for 30 s; 62°C for 45 s; 72°C for 1 min 30; 72°C for 10 min and 4°C), Miru 1, Locus 6, VNTR 19 (95°C for 2 min; 94°C for 1 min; 58°C for 1 min; 72°C for 1 min; 72°C for 10 min and 4°C) ST1 (95°C for 2 min; 94°C for 1 min; 65°C for 1 min; 72°C for 1 min; 72°C for 10 min and 4°C).

The PCR products were separated by electrophoresis on agarose gel containing 2% ethidium bromide (ETB). The visualization was carried out under UV light from an automated system (Gel documention, Bio-Rad Laboratories USA).

Primers used

The sequences of the primers used are summarized in Table 1.

Real time PCR

Amplification of IS2404 and Kr sequences was performed in the presence of specific primers for each sequence (Table 1) in a final reaction volume of 50 µL. For markers used, a PCR –mixer of 20 µl containing water for injection (H_2O ppi), 5X buffer, magnesium chloride (25 mM), dNTP, a detector of 10µM, a Rox Dye, 0.2µl DNA Taq polymerase (Hot Start Taq) and 5 µl of our DNA extract. The reaction mixture was incubated in a STEP ONE PLUS device as follows : 50°C for 2 min; 95°C for 10 min; 95°C for 15 S; 60°C for 1 min.

The control strain was *M. ulcerans* (ITM9540) provided by the molecular biology platform of Pasteur Institute of Côte d'Ivoire. The negative control was sterile distilled water.

RESULTS

A total of 473 samples were collected, 251 water and 222 sediment left based on sampling sites (Table 2). Based on morphological and biochemical characteristics, 65 strains (13.74%) were isolated and 17 species were identified (26.15%). From the water samples collected, 43.07% of species were identified, and 59.92% from sediments. These identified species were distributed according to the collection sites. 32.35% of the identified species are found in the hyper endemic sites, against 19.35% of species from hypo endemic sites. The *M. peregrinum* species, like *M. smegmatis*, *M. peregrinum*, *M. immunogenicum*, *M. chelonae*, *M. mucogenicum, M. abscessus, Mycobacterium* sp. were isolated in this study. *M. peregrinum* (13.84%) species was the most common in all sites, except in the sites of Bodo and Bouaké. The sediments are the most contaminated by *Mycobacteria* with 60% presence against 43.3% presence in water samples in hyper endemic areas. In hypo-endemic areas, water is the most contaminated with 53.57% presence against 43.24% in sediment samples. All species obtained were analyzed by PCR, focusing on IS2404 and Kr sequences. 9.23% of the isolated strains

Table 2. Samples collected according to sites and types.

Sample	Collection sites							
	Endemic sites				Non endemic sites			
	Adzopé	Adiopodoumé	Tiassalé		Agboville	Aghien	Bouake	Total
			Sokrogbo	Bodo				
Water	45	18	18	6	15	143	6	251
Sediments	16	18	18	6	15	143	6	222
Total	61	36	36	12	30	286	12	473

Table 3. Molecular characteristics of Identified mycobacteria species in each site.

Site	Samples collected	Mycobacteria species	IS6110	IS2404	Kr
Adzopé	Water	*M. smegmatis*	-	-	-
Adzopé	Water	*M. peregrinum*	+	-	-
Adzopé	Water	*M. mucogenicum*	+	-	-
Adzopé	Water	*M. mucogenicum*	-	+	+
Adzopé	Water	*M. mucogenicum*	-	+	+
Adzopé	Sediment	*M. peregrinum*	-	-	+
Abgoville	Sediment	*Mycobacterium sp.*	+	-	-
Abgoville	Sediment	*M. smegmatis*	-	-	+
Abgoville	Sediment	*M. immunogenicum*	-	-	-
Aghien	Sediment	*Mycobacterium peregrinum*	-	-	-
Aghien	Sediment	*Mycobacterium sp.*	+	-	-
Aghien	Water	*Mycobacterium sp.*	-	-	-
Aghien	Sediment	*Mycobacterium.peregrinum*	-	-	-
Sokrogbo	Sediment	*Mycobacterium sp.*	-	+	-
Sokrogbo	Water	*Mycobacterium sp.*	-	+	-
Sokrogbo	Sediment	*Mycobacterium sp.*	-	+	-
Sokrogbo	Sediment	*Mycobacterium sp.*	-	+	-
Bodo	Water	*M. mucogenicum*	-	+	-
Bodo	Water	*M. mucogenicum*	-	-	-
Adiopodoumé	Water	*M. peregrinum*	-	+	+
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	-
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	-	-
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	+
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	-
Adiopodoumé	Water	*Mycobacterium sp.*	-	+	-
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	-

IS2404: Insertion sequence found in *Mycobacterium ulcerans* and other environmental mycobacteria; Kr: synthesizing enzyme mycolactone toxin found in *Mycobacterium* ulcerans and other MPM. ; (-): Negative result in the desired sequence; (+): Positive result in the desired sequence. Positive control DNA (ITM9540); negative control: (H_2O).

had ketoreductase genes (KR), these two sequences were found in the fast-growing isolated species. *M. peregrinum, M. mucogenicum* and *Mycobacterium* sp. (Table 3). All strains were identified by using Miru-VNTR. The isolated strains showed different profiles in the presence of the MIRU1, VNTR19, LOCUS 6 and ST1. Of The 4 markers used, the MIRU 1 is the most amplified sequence, and LOCUS 6 less amplified. None known profile was identified in this study (Table 4).

DISCUSSION

Mycobacteria species are a large group distributed in

Table 4. Molecular typing of identified mycobacteria species according to sites.

Site	Mycobacteria specie	MIRU1	VNTR19	ST1	LOCUS 6
Adzopé	*M. smegmatis*	2	nd	nd	nd
Adzopé	*M. peregrinum*	nd	nd	2	nd
Adzopé	*M.mucogenicum*	4	nd	nd	nd
Adzopé	*M. mucogenicum*	nd	2	nd	nd
Adzopé	*M. mucogenicum*	nd	nd	1	nd
Adzopé	*M. peregrinum*	nd	nd	nd	nd
Abgoville	*Mycobacterium* sp.	nd	nd	nd	nd
Abgoville	*M. smegmatis*	4	nd	nd	nd
Abgoville	*M. immunogenicum*	3, 4	nd	nd	nd
Aguien	*Mycobacterium peregrinum*	nd	2	nd	nd
Aguien	*Mycobacterium* sp.	nd	nd	2	nd
Aguien	*Mycobacterium* sp.	nd	2	2	nd
Aguien	*Mycobacterium peregrinum*	nd	2	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Bodo	*M. mucogenicum*	nd	nd	nd	nd
Bodo	*M. mucogenicum*	nd	2	nd	1
Adiopodoumé	*M. peregrinum*	1	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	1, 2	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	1	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	nd	nd	2	nd
Adiopodoumé	*Mycobacterium* sp.	nd	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	4	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	1	nd	nd	nd

nd: Not determined ; Miru1 (1 copy, 2 copies, 3 copies and 4 copies); VNTR 19 (2 copies); ST1 (1 copy and 2 copies) ; LOCUS 6 (1 copy); the negative control (H_20) has a negative outcome for all PCR.

various aquatic and terrestrial environments. Most are saprophytic, but some species are pathogens capable of infecting humans and animals (Von Reyn et al., 1993; Falkinham, 2009). Their no specific pathogenic characteristic differentiates them from mycobacteria causing tuberculosis. The objective of this study was to make the molecular characterization of environmental mycobacteria strains in Côte d`Ivoire. The high sediment contamination identified in the study highlight the fast growing of mycobacteria (Kirschner et al., 1992). The risk of transmission of cutaneous mycobacteriosis would therefore also be present in non-endemic sites like in the endemic locations as demonstrated by Williamson et al. (2008) in the case of Buruli ulcer. The prevalence rate of environmental mycobacteria was 13.74%. This rate was relatively lower than the 15.5% observed by Kankya et al. (2011) in Ouganda. Parashar et al. (2004) also observed variations in the effectiveness of decontamination methods depending on the origin of the samples. Indeed it is known that mycobacterial species do not have the same resistance to different decontamination procedures (Parashar et al., 2004). According to the classification of Runyon, all isolated mycobacteria belongs to the group of fast growing mycobacteria (Group IV). The following species have been identified in this study: species *M. peregrinum, M. chelonae, M. abscessus, M. mucogenicum, M. immunogenum, M. smegmatis, M. peregrinum* and *Mycobacterium* sp. The species *M. peregrinum* was the most common in all sites, except in the sites of Bodo and Bouaké. All these species identified in Cote d`Ivoire are responsible for skin ulcerations in some countries as reported by Zamarioli et al. (2008) and Buijtels et al. 2009. *M. chelonae*, is responsible for skin soft tissue infections as reported by Sniezek et al. (2003), *M. abscessus* responsible for skin and soft tissue infections with abscess formation or skin nodules, often appearing after trauma or surgical operation with infected materials (Griffith et al. 2007). Among the isolated strains, 20% were carriers of the gene IS2404, they include *M. mucogenicum, M. peregrinum* and *Mycobacterium* sp.

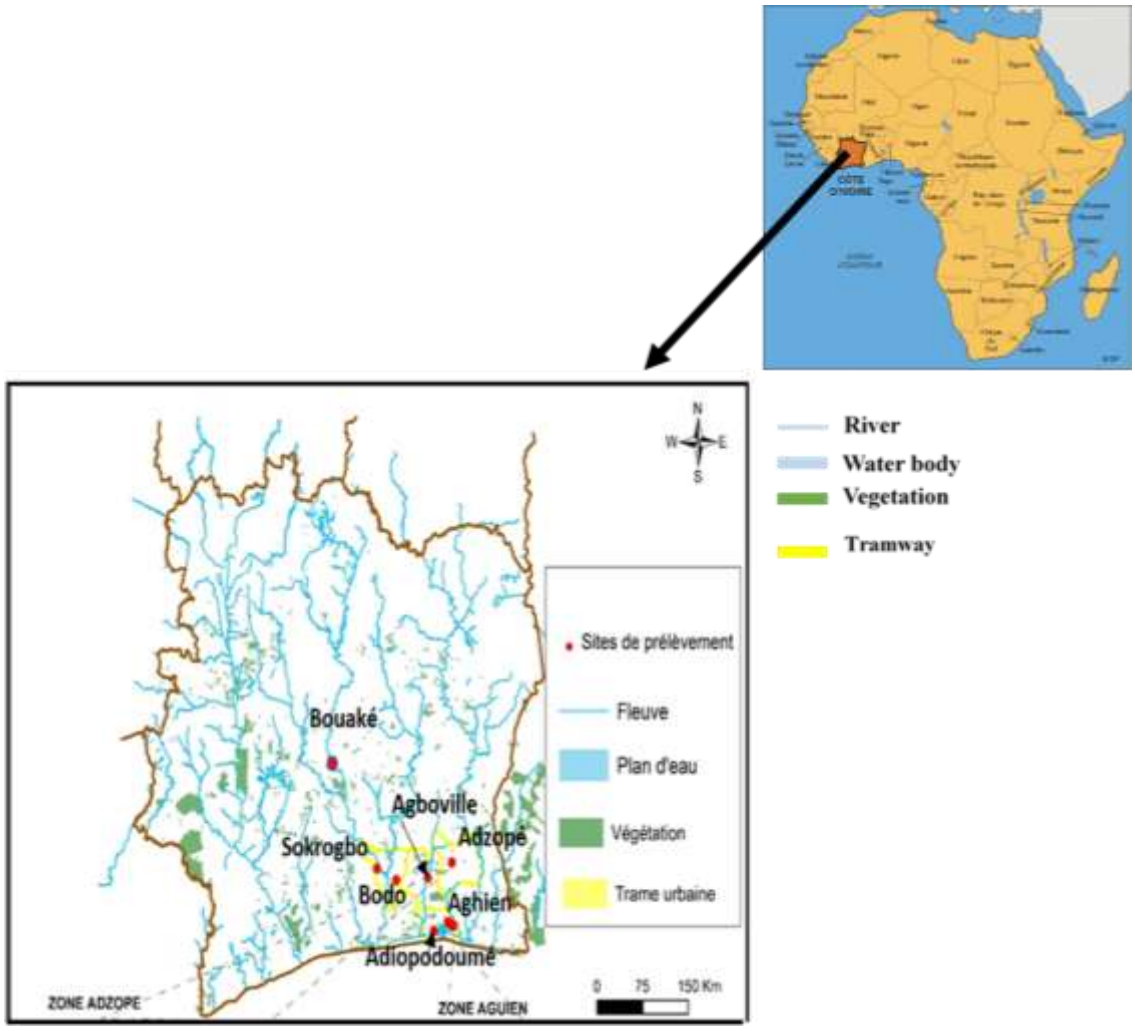

Figure 1. Sampling sites (Photo: Vakou Sabine, 2016).

This could imply the ability of these bacteria to cause skin ulcers in their isolated areas in Côte d'Ivoire. The sites of Bodo and Adzopé are known for their endemic zone to skin ulcers which has been attributed rightly or wrongly to *M. ulcerans* according to the National anti Buruli Ulcer Programe.

M. ulcerans is a slow-growing mycobacterium whose culture is often taken by default. The discovery in 1997 of the sequence of insertion IS2404 specific to *M. ulcerans* has been a catalyst for further research of the environmental germs (Ross et al. 1997). It shares this sequence with the other environmental mycobacteria such as *M. marinum, M. liflandii and M. pseudoshottsii* (Chemlal et al., 2002; Stragier et al., 2006). These slow growing mycobacteria would also be responsible for skin ulceration (Stragier et al., 2006). This sequence is an ideal for the diagnosis of Buruli ulcer (Portaels et al., 2009) could be good also for the diagnosis of Mycobacterial skin ulcerations.

Some differences were observed between the endemic sites and the non-endemic one. With the other level of contamination of water or sediments, the presence of *IS2404* gene was noticed only in the endemic sites. The Kr gene is most prevalent in endemic sites. This situation could be explain the sharing of genetic material between strains more frequently in endemic sites. But it could also be due to chance, in fact, Williamson et al. (2008), think that there would be no difference in the distribution of these genes between endemics and non-endemics sites. This study would be the first to reveal the existence of IS2404 gene in the fast-growing mycobacteria because according to previous studies, the IS2404 was the prerogative of slow growing mycobacteria (Chemlal et al., 2002; Stragier et al., 2006). In West Africa, Williamson et al. (2008), have also identified IS2404 gene in mycobacteria other than *M. ulcerans,* but they were the slow growing species. The ulcerations of Buruli ulcer are caused by one toxin, mycolactone (George et al., 1999).

A plasmid of 174 pb (pMUM001) present in the genome of the bacterium carries the polyketide synthetase genes (PKS), responsible for its' synthesis (Stinear et al., 2004, 2007). It was identified in other environmental mycobacteria that are slow growing, known to be mycolactone producing mycobacteria (Pidot et al., 2010). Results of this study also show its existence in the fast growing mycobacteria. Indeed, 9.23% of the isolated strains carry the ketoreductase gene (Kr), one of the enzymes involve in the synthesis of mycolactone according to Bali et al. (2006). Indeed, this enzyme would confirm the presence of the plasmid in the genome of the bacterium (Solange et al., 2015). Among the four specific markers, the MIRU 1 is the most amplified sequence, and LOCUS 6 the less amplified. No any known profile was identified in this study. These markers would be less appropriate to environmental isolated strains since they were tested only on samples from environmental and clinical strains (Stinear et al., 2007).

Conclusion

The results of this study highlight the potential risk of contamination in humans especially people in permanent contact with the environment. These species discovered, would be responsible for ulcerations in Côte d'Ivoire, which could explain the very high level of endemicity. It is therefore important to identify the sequences of these species in order to set up appropriate diagnostic methods. This study is just the first step, we want to understand the different skin infections encountered in Côte d'Ivoire. This will help to better diagnose patients suffering from skin infections other than Buruli ulcer and to consider strategies and means of protection of the population against all mycobacterioses by breaking the epidemiological chain.

In perspective, it would be good to continue studying the rapidly growing mycobacteria to determine their involvement in cutaneous ulcerations. The sequencing of the genes Kr and IS2404 isolated in the strains of this study for their eventual relationship with *M. ulcerans* could help for a better comprehension of the affection.

Conflict of interest

The authors declared that there is no conflict of interests.

REFERENCES

Ablordey A, Hilty M, Stragier P, Swings J, Portaels F (2005) Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in *Mycobacterium ulcerans.* J. Clin. Microbiol. 43**(**10):5281-5284.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987). Current Protocols in Molecular Biology, Greene Publishing Associates/Wiley Interscience, New York.

Bali S, Weissman KJ (October 2006). "Ketoreduction in Mycolactone Biosynthesis: Insight into Substrate Specificity and Stereocontrol from Studies of Discrete Ketoreductase Domains in vitro". Chem. BioChem. 7(12):1935-1942.

Barksdale L, Kim KS (1977): Mycobacterium. Bacteriol. Rev. 41:217

Buijtels PC, van der Sande MA, de Graaff CS, Parkinson S, Verbrugh HA, Petit PL (2009). Nontuberculous mycobacteria, Zambia. Emerg. Infect. Dis. 15:242-249.

Brown, Wallace (1992), Skin, soft tissue, and bone infections due to Mycobacterium chelonae: importance of prior corticosteroid therapy, frequency of disseminated infections, and resistance to oral antimicrobials other than clarithromycin. J. Infect. Dis. 166(2):405-12.

Caruso GFM, Passàli L, Salerni G, Molinaro M, Messina (2009). Head and neck mycobacterial infections in pediatric patients. Int. J. Pediatr. Otorhino laryngol. 73(1):38-41.

Chemlal K, Huys G, Laval F, Vincent V, Savage C, Gutierrez C, Laneelle MA, Swings J, Meyers WM, Daffe M, Portaels F (2002) Characterization of an unusual *Mycobacterium*: a possible missing link between *Mycobacterium marinum* and *Mycobacterium ulcerans*. J. Clin. Microbiol. 40(7):2370-2380.

Clovice K, Adrian M, Berit D, Musso M, John O-A, Eystein S, James O, Vigdis E, Tone BJ (2011). Isolation of non-tuberculous mycobacteria from pastoral ecosystems of Uganda: Public Health significance. BMC Public Health 11(1):1471-2458.

Dailloux M, Laurain C, Weber R, Hartemann P (2010). Water and non tuberculous mycobacteria. Water Res. 33:2219-2228.

del Rio Camacho G, Soriano Guillén L, Flandes Aldeyturriaga J,. Hernández García B, Bernácer Borja M (2010). Endobronchial atypical mycobacteria in an immunocompetent child. Pediatr. Pulmonol. 45(5):511-513.

Domenech P, Menendez MC, Garcia MJ (1994). Restriction fragment length polymorphisms of 16S rRNA genes in the differentiation of fast-growing mycobacterial species. FEMS Microbiol. Lett. 116(1):19-24.

Euzéby JP (2010). Techniques de diagnostiques en mycobactériologie. Dictionnaire de Bactériologie Vétérinaire. Available at: http:// www.mycobacterie.fr.

Falkinham JO (1996). Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. 9:177-215

Falkinham JO (2009). Surrounded by mycobacteria: Non tuberculous mycobacteria in the human environment. J. Appl. Microbiol. 107:356-367.

Fyfe JA, Lavender CJ, Johnson PD, Globan M, Sievers A, Azuolas J, Stinear TP (2007). Development and application of two multiplex real-time PCR assays for the detection of Mycobacterium ulcerans in clinical and environmental samples. Appl. Environ. Microbiol. 73(15):4733-4740.

George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, Small PL (1999). Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283:854-857.

Griffith DE, Aksamit T, Brown-Elliott BA, (2007). An official ATS/IDSA statement : Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am. J. Respir. Crit. Care Med 175:367-416.

Hilty M, Yeboah-Manu D, Boakye D, Mensah-Quainoo E, Rondini, S, Schelling E, Ofori-Adjei D, Portaels F, Zinsstag J. Pluschke G (2006). Genetic diversity in *Mycobacterium ulcerans* isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats. J. Bacteriol. 188:1462-1465.

Horsburgh CR. Jr. (1996). Epidemiology of disease caused by nontuberculous mycobacteria. Semin. Respir. Infect. 11(4):244-251

Inderlied CB, Kemper CA Bermudez LM (1993). The Mycobacterium avium complex. Clin. Microbiol. Rev. 6(3):266-310.

Kakou-Ngazoa E. Solange, Coulibaly N. David, AkaNguetta, Vakou Sabine Aoussi Serge and Dosso Mireille (2015) Clonality of *Mycobacterium ulcerans* by Using VNTR-MIRU Typing in

IvoryCoast (Côte d'Ivoire), West Africa. Int. J. Trop. Dis. Health 7(4):163-171.
Kirschner RA Jr, Parker BC, Falkinham JO III (1992): Epidemiology of infection by nontuberculous mycobacteria. *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* in acid, brown-water swamps of the southeastern United States and their association with environmental variables. Am. Rev. Respir. Dis. 145:271-275.
Kubica GP, Kaufmann AJ, Dye WE (1964). Comments on the use of the new mucolytic agent, N-acetyl-l-cysteine, as a sputum digestant for the isolation of mycobacteria. Am. Rev. Respir. Dis. 89:284-286.
Marsollier L, Robert R, Aubry J, Saint Andre JP, Kouakou H, Legras P, Manceau AL, Mahaza C, Carbonnelle B (2002). Aquatic insectsas a vector for *Mycobacterium ulcerans*. Appl. Environ. Microbiol. 68:4623-4628
Marsollier L, Stinear T, Aubry J, Saint-Andre´ JP, Robert R, et al. (2004) Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. Appl. Environ. Microbiol. 70:1097-1103.
Martin-Casabona N, Bahrmand AR, Bennedsen J (2004). Non-tuberculous mycobacteria : patterns of isolation. A multi-country retrospective survey. Int. J. Tuberc. Lung Dis. 8:1186-1193.
Menendez MC, Garcia MJ, Navarro MC, Gonzalez-y-Merchand JA, Rivera-Gutierrez S, Garcia-Sanchez L (2002). Characterization of an Rrna operon (*rrn*B) of *Mycobacterium fortuitum* and other mycobacterial species: Implications for the classification of mycobacteria. J. Bacteriol. 184(4):1078-1088.
Metchock B Nolte F. (1995): Mycobacterium. In: Murray PR,. Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds.): Manual of Clinical Microbiology, 6th ed. American Society for Microbiology, Washington, DC. pp. 400-437
Parashar D, Chaulan DS., Sharma VD, Chauhan A, Chauhan SVS, Katoch VM. (2004). Optimisation of procedures for isolation of mycobacteria from soil land waters amples obtained in northern India. Appl. Environ. Microbiol. 70:3751-3753
Pedley S, Bartram J, Rees G, Dufouret A Cotruvo JA (2004). Pathogenic mycobacteria in water: A guide to public healthconsequences, monitoring and management. S. Pedley, J.Bartram, G. Rees, A. Dufour and J. A. CotruvoEds. London, in World Health Organization titles with IWA Publishing. 1:1-229.
Pidot SJ, Asiedu K, Ka¨ser M, Fyfe JAM, Stinear TP (2010) Mycobacterium ulcerans and Other Mycolactone-Producing Mycobacteria Should Be Considered a Single Species. PLoS Negl. Trop. Dis. 4(7):e663.
Portaels F, Silva MT, Meyers WM (2009) Buruli ulcer. Clin Dermatol. 27:291-305.
Ross BC, Johnson PD, Oppedisano F, Marino L, Sievers A, Stinear T, Hayman JA, Veitch MG, Robins-Browne RM (1997). *Mycobacterium ulcerans* in environmental samples during an outbreak of ulcerative diseases. Appl. Environ. Microbiol. 63:4135-4138.
Runyon EH (1959): Anonymous mycobacteria in pulmonary disease. Med. Clin. N. Am. 43:273-290
Schiavone S, Coquery M (2011). Guide d'échantillonnage et de pré- traitement des sédiments en milieu continental pour les analyses physico-chimiques de la DCE. *Cemagref*, p24.
Sniezek PJ, Graham BS HB, Busch ER, Lederman ML, Lim K Poggemyer (2003). Rapidly growing mycobacterial infections after pedicures. Arch. Dermatol. Res. 139(1):629-634.
Solange KN, Coulibaly N, Sabine VA, Kouame KO, Serge AO, Constance KM, Helene KO, Julien AK, Raymond NG, Mireille DO (2015). Virulence factors expressed by *Mycobacterium ulcerans* strains: Results of a descriptive study Afr. J. Microbiol. Res. 9(28):1745-1751.
Stinear T, Ford T, Vincent V (2004). Analytical methods for the detection of waterborne and environmental pathogenic mycobacteria, p. 55-73. In. Pedley S, Bartram J, Rees, Dufour A, Cotruvo J (ed.), Pathogenic mycobacteria in water: a guide to public health consequences, monitoring and management. IWA Publishing, London, United Kingdom.
Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, (2007). Reduction evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. Genome Res. 17:192-200.
Stragier P, Ablordey A, Meyers WM, Portaels F (2005) Genotyping *Mycobacterium ulcerans* and *Mycobacterium marinum* by using mycobacterial interspersed repetitive units. J. Bacteriol. 187:1639-1647.
Stragier P., Ablordey A., Bayonne L. M., Lugor Y. L., Sindani I. S., Suykerbuyk P., Wabinga H., Meyers W. M., Portaels F. (2006) Heterogeneity among *Mycobacterium ulcerans* isolates from Africa. Emerg. Infect. Dis. 12:844-847.
Trujillo M, Velazquez E, Kroppenstedt RM, Schumann P, Rivas R, Mateos PF (2004). *Mycobacterium psychrotolerans* sp. nov., isolated from pond water near a uranium mine. International J. Syst. Evol. Microbiol. 54(5):1459-1463.
Von Reyn CF, Waddell RD, Eaton T, Arbeit RD, Maslow JN, Barber TW (1993): Isolation of Mycobacterium avium complex from water in the United States, Finland, Zaire, and Kenya. J. Clin. Microbiol. 31:3227-3230.
Winthrop KL, Abrams M. Yakrus MA, Schwartz I, Ely J, Gillies D, (2002). An outbreak of mycobacterial furunculosis associated with footbaths at a nail salon. New Engl. J. Med. 346(18):1366-1371.
Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, et al. (2008) Distribution of *Mycobacterium ulcerans* in Buruli Ulcer Endemic and Non-Endemic Aquatic Sites in Ghana. PLoS Negl. Trop. Dis. 2(3):e205.
Wilton S, Cousins D. (1992) Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube. PCR Methods Appl. 1:269-273
Ziza JM, Desplaces N (2006). Infections ostéoarticulaires à mycobactéries atypiques. Revue du rhumatisme 73: 394-400.
Zamarioli LA, Coelho AG, Pereira CM, Nascimento AC, Ueki SY, Chimara E (2008). Descriptive study of the frequency of non tuberculous mycobacteria in the Baixada Santista region of the state of Sao Paulo, Brazil. J. Bras. Pneumol. 34(8):590-594.

academicJournals

Vol. 10(40), pp. 1711-1714, 28 October, 2016
DOI: 10.5897/AJMR2016.8026
Article Number: 4E0277261342
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Efficacy of biocontrol agents in the management of head rot of cabbage (*Brassica oleracea var. capitata*) caused by *Sclerotinia sclerotiorum*

Kamesh Krishnamoorthy K.*, Sankaralingam A. and Nakkeeran S.

Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore-641003, India.

Received 24 March, 2016; Accepted 26 April, 2016

Head rot of cabbage caused by *Sclerotinia sclerotiorum* leads to rotting of fully matured cabbage heads in the field. In the present study the antagonistic effects of twenty *Bacillus* isolates was tested against *S. sclerotiorum in vitro*. Eight effective *Bacillus* isolates obtained from studies *in vitro*, commercial formulations of *Trichoderma viride* isolate TV-1 and *Pseudomonas fluoroscens* isolate Pf-1along with a fungicide check (Nativo-Tebuconazole+Trifloxystrobin) were carried further for field studies. Results of field studies indicated that fungicide check of Nativo (1.5 g/L) was highly effective with least disease incidence of 10.36% indicating 74.50% reduction over control. Among the biocontrol agents commercial formulation of *Trichoderma viride* isolate TV-1 was the most effective showing disease incidence of 11.38% indicating 72.00% reduction over control followed by *Bacillus amyloliquefaciens* isolate B15 and *Pseudomonas fluoroscens* isolate Pf-1 showing disease incidence of 13.24 and 13.31% indicating 67.41 and 67.24% reduction over control respectively and both treatments were on par. *B. licheniformis* isolate B16 was found to be least effective with 20.41 percent disease incidence indicating 49.76% reduction over control.

Key words: Bacillus, commercial formulation, fungicide check, *Pseudomonas fluoroscens, Trichoderma viride, Sclerotinia sclerotiorum.*

INTRODUCTION

Head rot of cabbage caused by the pathogen *Sclerotinia sclerotiorum* leads to rotting of fully grown cabbage heads in the field and during post-harvest operations and storage (Hudyncia et al., 2000). The pathogen is geographically cosmopolitan and has a broad ecological distribution (Purdy, 1979). The broad host range of the pathogen includes high value crops like alfala, bean, cabbage, canola, lettuce, peanut, soybean, sugarbeet, sunflower, tobacco and tomato (Grau, 1988; Farr et al., 1989).

The relatively unreliable control of *S. sclerotiorum* with traditional methods and concerns about pesticide residues has prompted interest in biological control as an alternative disease management strategy (Fernando et

*Corresponding author. E-mail: kamukrishagri@gmail.com. Tel: - 9944705207.

al., 2007). Biological control of S*. sclerotiorum* has received considerable attention as an alternative disease management tactic to the use of fungicides due to its ability to provide safe and environmentally friendly disease control (Xiaojia et al., 2013).

Attributes of *Bacillus* spp. such as high thermal tolerance and ready formulation of endospores makes it an ideal agent for the development of commercial products. They adhere firmly to root surface especially when an inoculum of spore is used (Shoda, 2000). *Bacillus* spp. are known to survive well under field conditions due to the production of endospores (Boyetchko et al., 1999; Collins and Jacobsen, 2003). *Bacillus* spp. isolated from rhizosphere of common bean were tested for their antifungal activities against *S. sclerotiorum*. The maximum inhibition in radial growth caused by *Bacillus* spp.BPR7 was observed after seven days of incubation in dual culture and cell free culture filtrate (Pankaj et al., 2012). Fernando et al. (2013) reported that the metabolites produced by *B. subtilis* were antagonistic to the fungus with 17.7% reduction in mycelial growth which was constant even after two weeks suggesting high efficiency of the metabolites in control of *S. sclerotiorum*.

The aim of the present study was to determine efficacy of biocontrol agents under *in vitro* and field conditions.

MATERIALS AND METHODS

Screening of *Bacillus* isolates *in vitro*

Standard isolates of *Bacillus* spp. maintained as glycerol stocks were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The isolates were subcultured and maintained on Nutrient agar (NA) medium for further studies. The antagonistic effects of twenty *Bacillus isolates* were tested against *S. sclerotirum* by dual culture technique. A 9-mm-dia mycelial disc of the pathogen was placed at one end of the Petri plate containing combination medium (Potato Dextrose Agar + Nutrient Agar) and the bacterial antagonist was streaked at the opposite end. Inoculation of the pathogen without antagonist served as control and each treatment was replicated three times. The plates were kept in an incubator at 20±2°C. When the fungus attained full growth in the control plate, growth of the pathogen and inhibition zone were measured and percent reduction in growth over control was calculated.

Percent inhibition over control was calculated using the formula:

$$\frac{C - T}{T} \times 100$$

Where C = growth of *S. sclerotiorum* in control; T = growth of *S. sclerotiorum* in treatment.

Commercial formulations of *Trichoderma viride* and *Pseudomonas fluoroscens*

The commercial formulations of *Trichoderma viride* (TV-1) as talc formulation and *Pseudomonas fluoroscens* (Pf-1) as liquid formulation were obtained from *Trichoderma* Lab and *Pseudomonas* Lab respectively from Department of Plant Pathology, Centre for Plant Protection Studies (CPPS), Tamil Nadu Agricultural University , Coimbatore, Tamil Nadu, India.

Field trial

Eight effective *Bacillus* isolates obtained from *in vitro* studies, commercial formulations of *Trichoderma viride* (TV-1) and *Pseudomonas fluoroscens* (Pf-1) along with a fungicide check (Nativo-Tebuconazole+Trifloxystrobin) were carried further for field studies.

The field trial was conducted between February and March 2014 in the Kothagiri area of Nilgiris district and was laid out in a randomised block design. First spraying was done 48 days after planting when the cabbage was at cupping stage and subsequent sprayings were done at 7 days interval till head fill stage of the cabbage.

There were nine treatments in all consisting of *Bacillus amyloliquefaciens* (B15), *B. licheniformis* (B 19), *B. cereus (*B31), *B. licheniformis (*BSD-1), *B. subtilis* (B14), *B. licheniformis (*B16), *Pseudomonas fluorescens* (Pf-1) (commercial formulation), *Trichoderma viride* (TV1) (commercial formulation) each at a concentration of 10 mL/L and Fungicide check- Nativo (Tebuconazole+Trifloxystrobin) at a concentration of 1.5 g/L. The control had no bio control agent treatment at all.

The percent disease incidence was calculated using the following formula:

$$\frac{\text{Number of infected plants}}{\text{Total number of plants observed}} \times 100$$

Results were expressed in terms of per cent disease reduction over control which was calculated as follows:

$$R = \frac{100\ (C\text{-}T)}{C}$$

Where, R = Percent reduction over control; C = Percent disease incidence in control; T = Percent disease incidence in treatment.

Data analysis method

Statistical analysis was performed using the IRRISTAT software version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). Before performing the statistical analysis of variance (ANOVA) the percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$) and means were separated by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Twenty *Bacillus* isolates were tested *in vitro* against *S. sclerotiorum*. The treatment B15 (*B. amyloliquefaciens*) yielded the minimum mycelial growth of 53.0 mm and the highest growth reduction of 41.0% with an inhibition zone of 26.6 mm (Table 1). This was followed by B19 (*B. licheniformis*) which recorded 54.3 mm growth with39.7% growth reduction with an inhibition zone of 24.3 mm. However both the treatments were at par with each other.

Table 1. Effect of *Bacillus* isolates on the growth of *S. sclerotiorum* under *in vitro.*

Isolates	Mycelial growth (mm)	Inhibition over control (%)	Inhibition zone (mm)
B31-*Bacillus cereus*	55.0[a]	39.0	23.6[a](4.90)
B14-*B.subtilis*	62.3[bc]	30.7	17.3[b](4.22)
B19-*B.licheniformis*	54.3[a]	39.7	24.3[a](4.98)
B12-*B.licheniformis*	65.3[cd]	27.7	14.6[bc](3.89)
BSC-2-*B.tequilensis*	71.0[ef]	21.0	0.0[f] (0.70)
BSC-3-*B.subtilis*	73.0[efg]	19.0	0.0[f](0.70)
BSC-6-*B.amyloliquefaciens*	74.7[fg]	17.0	0.0[f](0.70)
BSC-7-*B.amyloliquefaciens*	65.0[cd]	27.7	7.13[d](2.76)
B18-*B.megaterium*	73.3[efg]	18.8	0.0[f](0.70)
B15-*B.amyloliquefaciens*	53.0[a]	41.1	26.6[a](5.21)
B16-*B.licheniformis*	62.7[bc]	30.3	6.6[d](2.67)
B30-*B.subtilis*	69.0[de]	23.3	8.57[d](3.01)
B2-*B.amyloliquefaciens*	74.0[fg]	17.7	0.0[f](0.70)
Bag3-*B.megaterium*	76.7[g]	14.7	0.0[f](0.70)
B11-*B.licheniformis*	87.7[h]	2.3	0.0[f](0.70)
B17-*B.megaterium*	72.0[efg]	20.0	3.50[e](2.00)
BSc-9-*B.amyloliquefaciens*	76.0[g]	15.7	0.0[f](0.70)
BSD-1-*B.licheniformis*	60.0[b]	33.3	11.7[c](3.50)
B3-*B.subtilis*	74.3[fg]	17.7	0.0[f](0.70)
BSC-5-*B.cereus*	72.3[efg]	19.7	0.0[f](0.70)
Control	90.0[h]	-	0.0[f](0.70)

[1]Figures in parenthesis are square root transformed values; [2]In a column means followed by same letter are not significantly different at the 5% level of DMRT.

Table 2. Efficacy of biocontrol agents under field conditions for the management of *Sclerotinia* head rots of cabbage.

T. No.	Treatments	Disease incidence (%)	Reduction over control (%)	Yield (t/ha)
T_1	B15-*Bacillus amyloliquefaciens* at 10ml/L	13.24[c](21.34)	67.41	44.68[c]
T_2	B19- *B. licheniformis* at10ml/L	15.50[d](23.18)	61.85	41.64[d]
T_3	B31- *B. cereus* at10ml/L	16.50[e](23.96)	59.38	40.71[e]
T_4	BSD-1-*B. licheniformis*at10ml/L	18.57[f](25.52)	54.29	40.69[e]
T_5	B14-*B. subtilis*at10ml/L	19.10[g](25.91)	53.00	40.44[f]
T_6	B16-*B. licheniformis*at10ml/L	20.41[h](26.85)	49.76	40.31[g]
T_7	Pf1-*Pseudomonas fluorescens* (commercial formulation) at 10 ml/L	13.31[c](21.39)	67.24	44.56[c]
T_8	TV-1-*Trichoderma viride* (commercial formulation) @ 10g/L	11.38[b](19.71)	72.00	46.34[b]
T_9	Fungicide check -Nativo (Tebuconazole+Trifloxystrobin)@1.5g/L	10.36[a](18.77)	74.50	47.86[a]
T_{10}	Control	40.63[i](39.60)	-	37.45[h]

Figures in paranthesis are arc sine transformed values; In a column means followed by same letter are not significantly different at the 5% level of DMRT.

B. amyloliquefaciens inhibited growth of *S. sclerotiorum in vitro* which was indicated by an inhibition zone between the two organisms (Abdullah et al., 2008). Strains of *B. amyloliquefaciens* $ARP_2$3 and $MEP_2$18 caused alterations in sclerotial morphology and sclerotial germination of *S. sclerotiorum* causing stem rot of soybean according to Alvarez et al. (2012). Under *in vitro* *B. licheniformis* strain 9555 showed effective antifungal activity against *S. sclerotiorum* (Vipin et al., 2012).

Results of field studies indicated that fungicide check of nativo applied at a concentration of 1.5 g/L was highly effective with least disease incidence of 10.36% indicating 74.50% reduction over control (Table 2). Among the biocontrol agents commercial formulation of

T. viride isolate (TV-1) was the most effective treatment showing disease incidence of 11.38% indicating 72.00% reduction over control. This was followed by *B. amyloliquefaciens* isolate (B15) and *P. fluorescens* isolate (Pf-1) showing disease incidence of 13.24 and 13.31% indicating 67.41 and 67.24% reduction over control respectively and both treatments showed no significant difference. *B. licheniformis* isolate (B16) was found to be least effective with 20.41% disease incidence indicating 49.76% reduction over control. Maximum yield of 47.86 t/ha was observed for the fungicide check - Nativo treatment (Table 2). This was followed by the *T. viridae* (TV-1) treatment with 46.38 t/ha yield. Gaur et al. (2010) reported that under field conditions *Sclerotinia* stem rot of mustard caused by *S. sclerotiorum* was effectively controlled by seed treatment (10g/kg) and foliar spray (0.2 per cent) at 50 days after sowing with talc based mixed formulation of *T. hamatum* and *T. viridae* in the ratio of 1:1 followed by bioagent combination of *T. harzianum* (10 g/kg) and *Gliocladium virens* (0.2%) over two consecutive years. *P. fluorescens* isolate (P13) decreased severity of *Sclerotinia* stem rot of oilseed rape by 59% under field conditions and also promoted seedling growth (Li et al., 2011). Use of *B. amyloliquefaciens* as a soil treatment supressed stems rot of cucumber caused by *S. sclerotiorum* (Sharie et al., 2013).

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Abdullah MT, Ali NY, Patrice S (2008). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary with *Trichoderma harzianum* and *Bacillus amyloliquefaciens.* Crop Prot. 27:1354-1359.

Alvarez F, Castro M , Príncipe A, Borioli G, Fischer S, Mori G, Jofré E (2012).The plant-associated *Bacillus amyloliquefaciens* strains $MEP_2$18 and $ARP_2$3 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of *Sclerotinia* stem rot disease. J. Appl. Microbiol. 112:159-174.

Boyetchko S, Pedersen E, Punja Z, Reddy M (1999). Formulations of biopesticides. Methods Biotechnol. 5:487-508.

Collins DP, Jacobsen BJ (2003). Optimizing a *Bacillus subtilis* isolate for biological control of sugarbeet cercospora leaf spot. Biol. Control 26:153-161.

Farr DF, Bills GF, Chamuris GP, Rossman AY (1989). Fungi on Plants and Plant Products in the United States. In. The American Phytopathological Society. P 1252.

Fernando PM, Larissa CF, Leandro PE, Paulo, ES (2013). Antagonism of *Bacillus subtilis* against *Sclerotinia sclerotiorum* on *Lactuca sativa*. J. Agric. Sci. 5(4):214-223.

Fernando WGD, Nakkeeran S, Zhang Y, Savchuk S (2007). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Prot. 26(2):100-107.

Gaur RB, Sharma RN, Gautam VS, Dangi RP (2010). Management of *Sclerotinia* rot of mustard through bioagents. Indian Phytopathol. 63:392-397.

Gomez KA, Gomez AA (1984). Statistical Procedure for Agricultural Research. John Wiley and Sons, New York.

Grau CR (1988). Soybean diseases of the north central region. In. American Phytopathological Society. pp. 56-66.

Hudyncia J, Shew HD, Cody BR, Cubeta MA (2000). Evaluation of wounds as a factor to infection of cabbage by ascospores of *Sclerotinia sclerotiorum*. Plant Dis. 84(3):316-320.

Li H, Li H, Bai Y, Wang J, Nie M, Li B, Xiao M (2011). The use of *Pseudomonas fluoroscens* P13 to control Sclerotinia stem rot (*Sclerotinia sclerotiorum*) of oilseed rape. J. Microbiol. 49(6):884-889.

Pankaj K, Dubey RC, Maheshwari DK (2012). *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiol. Res. 167:493-499.

Purdy LH (1979). *Sclerotinia sclerotiorum*:History, diseases and symptomatology, host range, geographic distribution and impact. Phytopathology 69:875-880.

Sharie R, Mojdeh M, Dariush S (2013). The use of *Bacillus amyloliquefaciens* to control *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*) of cucumber. Int. J. Farm. Allied Sci. 2:965-970.

Shoda M (2000). Bacterial control of plant diseases. J. Biosci. Bioeng. 89:515-521.

Vipin MD, Sandhya M, Vasvi C, Swati T, Poonam S, Sumit Y, Shashank M, Ijinu TP, Varghese G, Ajit V, Chandrashekhar N (2012). Biocontrol and plant growth promotion and conferring stress tolerance: Multifaceted role of *Bacillus licheniformis* 9555. J. Fitopatolgi 3(4):780-783.

Xiaojia H, Daniel PR, Lihua X, Jude EM, Changbing Y, Yinshiu L, Shujie Z, Xing L (2013). *Bacillus megaterium* A6 supresses *Sclerotinia sclerotiorum* on oilseed rape in the field and promotes oilseed rape growth. Crop Prot. 52:151-158.

academicJournals

Vol. 10(40), pp. 1715-1719, 28 October, 2016
DOI: 10.5897/AJMR2016.8239
Article Number: 779255D61347
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effect of gamma irradiation (Co60) in the control of *Campylobacter* sp. in chilled chicken (*Gallus gallus*) heart

Marta Maria Braga Baptista Soares Xavier[1]*, Robson Maia Franco[1], Ana Beatriz Monteiro Fonseca[2], Edgar Francisco Oliveira de Jesus[3], Mauro Carlos Lopes Souza[3], Sheila da Silva Duque[4], Wagner Thadeu Cardoso Esteves[4]

[1]Universidade Federal Fluminense (UFF). Faculdade de Veterinária. Rua Vital Brazil Filho, 64. Niterói-RJ. CEP: 24230-340. Brazil.
[2]Universidade Federal Fluminense (UFF). Faculdade de Matemática. Niterói, RJ, Brazil.
[3]Universidade Federal do Rio de Janeiro (UFRJ). Ilha do Fundão, RJ, Brazil.
[4]Coleção de *Campylobacter*. Laboratório de Zoonoses Bacterianas CCAMP. IOC. FIOCRUZ. RJ, Brazil.

Received 29 July 2016; Accepted 2 September, 2016.

The present study aimed to evaluate the efficiency of the irradiation process in the control of *Campylobacter* spp. in chilled chicken heart samples, since this microorganism is related to the contamination of meat and chicken giblets and is responsible for enteritis in humans. The methodology and standards recommended by RDC no. 12 (Brazil, 2001) were applied for the bacteriological analyses. The chilled chicken heart samples were acquired in an industry that undergoes sanitary inspection, located in the West Zone of Rio de Janeiro. Samples were divided into two groups, non-contaminated (NC - originally from the industrial plant) and contaminated (CAMPY– contaminated with *C. jejuni* ATCC 33291/CCAMP/FIOCRUZ 00262 strains by CCAMP/LABZOO/IOC/FIOCRUZ), subsequently separated into four groups: NC and CAMPY control groups and samples irradiated at 1.5, 3.0 and 4.5 kGy. The eight subgroups were analyzed for the presence of *Campylobacter* sp. No statistically significant difference was observed between the four groups namely the non-irradiated controls and the 1.5, 3.0 and 4.5 kGy irradiated samples (p> 0.05). Elimination of *Campylobacter* sp. was observed, with no bacterial growth in any of the irradiated, non-contaminated (CN) and infected (CAMPY) samples. Thus, the efficiency of the Co60 irradiation process of chilled chicken heart for the elimination of the surveyed microorganisms was proven. The lowest dose applied was sufficient to eliminate the enteric pathogen which is of great significance in a public health point of view. However, it should be noted that the Brazil legislation determining the microbiological standards for food does not include a microbiological standard for *Campylobacter* sp. This means that any amount of this enteric pathogen may bring public health risks.

Key words: Food irradiation; chicken heart; *Campylobacter* sp.; Foodborne illness; public health.

INTRODUCTION

Food irradiation is a proven safe storage physical method, considered cold pasteurization, in which the food is exposed to a defined dose of ionizing radiation. Improvements in the microbiological quality of the product

are observed with irradiation, reducing the risk of foodborne illness along with decreased losses in storage and longer shelf life. This method does not influence the appearance and composition of nutrients, and its main objective is food security. However, the great challenge when applying this method is consumer acceptance, often due to confusion of “irradiated” with “radioactive” (Diehl, 1995; Hernandez et al. 2003; Gava, 2006; Miranda; 2012).

Brazil’s poultry industry began the year of 2016 beating several records, including the chickens’ production and exports. Chicken meat, is consolidated as the fourth item of the national export portfolio, which achieved the three best monthly results in the history of the sector's exports in, 2015 (Brazilian Association of Animal Protein, 2015). The importance of studying giblet and chicken meat contamination is highlighted by the fact that, these products are important source of high quality protein rich in essential amino acids, vitamins and minerals, and highly consumed not only in Brazil but throughout the world (Poultry Brazil, 2012). However, the Brazilian health legislation determining the microbiological standards for food, RDC Resolution no.12 (Brasil, 2001) does not state any maximum permissible limit as microbiological criteria regarding the presence of *Campylobacter* sp. in meat and chicken giblets, as it does for other pathogens in Annex II of this standard.

There is the likelihood of these organisms that have been noted during the various stage of animal raising, transport, processing, distribution and marketing, and serve as indicators of the sanitary conditions of the production/handling of raw materials, since they can be responsible for foodborne illness (Clements, 2011; Franco, 2012; Russel, 2009). The present study aimed, to evaluate the effects of gamma radiation (Co60) on the microbiological quality and control of *Campylobacter* sp. in chilled chicken (*Gallus gallus*) heart samples.

MATERIALS AND METHODS

Collection of samples

Chicken heart samples were acquired from a poultry slaughter house that undergoes regular sanitary inspection and has an on-site store for meat sales, located in the western zone of the state of Rio de Janeiro, Brazil. Three samplings were conducted. Giblets were randomly selected taking into account the production date, closest to the beginning of the analysis. The samples were placed in an isothermal container and kept under refrigeration during all stages of the experiment, at a maximum temperature of 7°C (Brasil, 1996). Samples were then distributed in previously identified Zip lock bags (contaminated – CAMPY, non-contaminated - NC, chicken heart - CF, control – non- irradiated samples, and irradiated samples – with 1.5kGy, 3.0kGy and 4.5kGy) with the corresponding date of the analysis. Contaminated aliquots received a prepared homogenized bacterial suspension containing 9.0 mL of 0.1% TPA and mass generated from a *C. jejuni* ATCC 33291/CCAMP 0262 strains seed culture, provided by the *Campylobacter* Bacterial Zoonosis Laboratory, at the Oswaldo Cruz Institute, Oswaldo Cruz Foundation (Rio de Janeiro, RJ, Brazil). Turbidity caused by bacterial growth was at a McFarland scale #1, equivalent to 3.0 x 10^{-8} bacteria mL^{-1} (Bier, 1980).

Irradiation of the samples

The non-contaminated and contaminated samples were then transported to the Nuclear Instrumentation Laboratory at, Alberto Luiz Coimbra Institute of Graduate Studies and Research in Engineering (COPPE), in Federal University of Rio de Janeiro, in an isothermal container, where they were subjected to gamma irradiation process (Co60) at the dosage of 1.5kGy, 3.0kGy and 4.5kGy. The control samples (NC and CAMPY) were not irradiated and remained in the isothermal container throughout the irradiation process of the other samples (Caruso et al., 2011).

Cultivation of *Campylobacter* sp.

For cultivation, identification and maintenance of *Campylobacter* sp. strains, a standard technique was implemented at the Bacterial Zoonoses Laboratory (Filgueiras and Hofer, 1989). The selective medium comprising a nutrient base (4.4 g Columbia agar, 0.4 g activated carbon diluted in 100 mL distilled water) was prepared by adding an FBP supplement as an oxygen-reducing substance (0.5g ferrous sulfate, sodium bisulfite and sodium pyruvate, diluted in 100 mL sterile distilled water) and an antimicrobial mixture (11mg cephalothin, 50mg trimethoprim lactate, 91 mg vancomycin, 20mg actidione, and 22 mg colistin, diluted in 50 mL sterile distilled water). The media was poured into 20 plates (five replicates for each NC and CAMPY sample) and stored in a GasPak jar (in a microaerophilic atmosphere with anaerocult® sachets). The inoculated plates were incubated at 42°C for 48 h.

Campylobacter sp. colonies were isolated after confirming typical morphotinctorial characteristics and subjected to Gram staining. Replating was performed in plates with selective media for mass formation. NC and CAMPY plates were analyzed in control and irradiated samples. The replating was performed to obtain mass formation, to have colonies to realize biochemical tests at the end of the experiment. Both NC and CAMPY plates were analyzed because on the NC plates there was no certainty of finding *Campylobacter sp*. and the samples showed original slaughter house poultry microorganisms, which certainly occured in the CAMPY plates groups. Being a contaminated sample, it is a known fact that microorganisms would be present, and there is need to ascertain the gamma radiation effect on the control of these microorganisms. Therefore, the two groups were evaluated.

Identification of *Campylobacter* sp.

Tests such as the hydrolysis of Na hippurate was performed to be confirm as well as to differentiate genus/species (Lior, 1982) Characterization of *Campylobacter* genus was also conducted since there are differences between *Campylobacter jejuni* and *Campylobacter coli* (the former produces glycine and

*Corresponding author. E-mail: m2b2sx@hotmail.com.

Table 1. *Campylobacter* sp. control in chilled chicken heart.

Group	Doses (kGy)	Week 1					Week 2					Week 3				
		A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
NC	C	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CAMPY	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NC – Non-contaminated samples; CAMPY – Contaminated samples. A, B, C, D and E correspond to the five plates used for each dose.

forms a purple halo on the tube surface while the latter does not produce glycine and produces a colorless halo on the surface of the tube). Indoxil acetate hydrolysis was also verified; the results were interpreted as negative when no change in disk coloring occurred, and as positive when the disk became blue-green/dark blue, indicating the presence of *C. jejuni* and *C. coli*. To complete the biotyping, the presence of deoxyribonuclease enzyme. (DNAse) was also determined using the methyl green agar DNAse test, evidence of enzyme activity on the substrate was obtained by observing the presence of a pink/salmon halo At the end of the experiment, 15 confirmed strains were deposited in the *Campylobacter* collection/ Bacterial Zoonosis Laboratory (IOC/ FIOCRUZ/ RJ/ Brazil).

Statistical analysis

Statistical differences between groups were evaluated by applying Friedman's test, with a significance level set at 0.05.

RESULTS AND DISCUSSION

No significant difference was observed between the three samplings, regardless of group (NC or CAMPY) and dosage subgroup (control, 1.5, 3.0 and 4.5 kGy), indicating no effect on the positive results for the presence of *Campylobacter* sp. (p=0.444). This is probably caused by the fact that the positive samples for *Campylobacter* sp. were the control samples, not subjected to the irradiation process, confirming the occurrence of *Campylobacter* sp. in meat and chicken giblets, as reported by Azeredo et al. (2010), Campos et al. (2015), Freitas and Noronha (2007).

The results of Co60 gamma radiation process in chilled chicken heart samples exposed to different radiation doses are displayed in Table 1.

During the first week, of the five control plates seeded with the *in natura* samples (NC), two (A and D) were positive (40%), whereas the contaminated samples (CAMPY) were all positive (100%). For the samples irradiated at 1.5kGy, 3.0kGy and 4.5kGy, both the non-contaminated (NC) and contaminated (CAMPY) samples showed no cell growth (100%), demonstrating the efficiency of the Co60 gamma irradiation process in the control of *Campylobacter* sp. The result corroborates with the reports of Ahn et al. (2013) which states that irradiation is an effective technological process to eliminate pathogens in poultry meat. In addition, an advantage of ionizing Co60 radiation includes high penetration and uniform dosage. In concordance with the results of this study, Mendonça (2002), Olson (1998) and Raut et al. (2012) reported that the majority of the mundane enteric pathogens such as *Campylobacter jejuni*, can be significantly reduced or eliminated with low dose (<3.0 kGy) irradiation.

During the second week, control plates A, B, C and D were positive (80%). However, both NC and CAMPY samples irradiated with all three doses did not demonstrate colony growth, and the process was thus effective in eliminating *Campylobacter* sp. Raut et al. (2012) which evaluate the effectiveness of the irradiation process in the elimination of *Campylobacter* sp. by, testing the sensitivity of *Campylobacter jejuni* and *Campylobacter coli* in chicken meat samples at doses ranging from 0.110 to 0.190 kGy. These authors demonstrated that, treatment with a dosage of 1kGy can achieve complete elimination of *Campylobacter* sp. in poultry meat samples and the results completely justifies the elimination of microorganism using 1.5kGy irradiation process in both NC and CAMPY samples of this study.

In the third and final week of the experiment, control samples A, B, D and E were positive (80%), while all irradiated samples showed no development of colonies (100%). Kudra et al. (2012) compared the efficacy of irradiation process in the control of *Campylobacter jejuni* in chicken breasts to that of modified atmosphere packaging (MAP). The D_{10} sensitivity value regarding irradiation of this enteric pathogen ranged from 0.31+0.01 kGy in vacuum packaging, and 0.29+0.03kGy in MAP, respectively. Irradiation was effective in eliminating *C. jejuni* from chicken breast packed both in vacuum or MAP, thereby reducing the possibility of cross-

contamination in retail shops or in domestic kitchens. The doses used in the present study were higher and reached their goal regarding the control of *Campylobacter* sp. Therefore, additional means to mitigate quality changes appear to be required for these products.

Comparable to the results obtained in the present study, Chun et al. (2010) and Haughton et al. (2012) investigated the applicability of UV-C irradiation in the inactivation of *Campylobacter jejuni* in ready-to-eat poultry and chicken fillets, respectively, and their results demonstrated the control of this microorganism during storage using this type of irradiation. The presence of *Campylobacter* sp. regardless of the dosage subgroup (control, 1.5, 3.0 and 4.5 kGy) were compared among the NC groups, and no significant difference between the three samplings was observed, with no effect regarding positive results for the presence of this microorganism *(*p=0.444). The presence of *Campylobacter* sp. in chilled chicken heart samples was evidenced in the present study, regardless of the type of treatment, similar to other studies that observed the presence of this microorganism derived from feces and meat and chicken giblets (Bognar, 2012; Trassi, 2012). Also, in regardless of the dosage subgroup (control, 1.5, 3.0 and 4.5 kGy) was also compared among the CAMPY groups, and again no significant difference between the three samplings was observed, with no effect regarding positive results for the presence of this microorganism *(*p=1.000).

As in the present study, Azeredo (2007), in his experiment on irradiated chicken livers with doses of 0.20kGy, 0.27, 0.30 and 0.35, also used two groups, one non-contaminated and one contaminated with *Campylobacter* sp. and concluded that there was no significant difference between the contaminated samples and those with other treatments. Clavero et al. (1994) observed ground beef samples contaminated with *Campylobacter jejuni* which were subjected to irradiation treatment with Co60 gamma doses ranging from 0 to 2.52kGy, and observed significant values depending on the combination performed in the experiment, of temperature and fat content. The authors concluded that, regardless of the selected treatment, pathogens were highly sensitive to gamma irradiation, and a D_{10} value for *Campylobacter jejuni* was determined ranging from 0.175 to 0.235kGy.

The authors also observed that, a 2.5kGy dose would be sufficient to eliminate $10^{10.6}$ *Campylobacter jejuni*, resulting in a high probability of complete inactivation of much higher populations than those occasionally present in preparations with ground meat. Patterson (2008) investigated the sensitivity of different *Campylobacter* species to irradiation in poultry samples and reported D_{10} values ranging from 0.12 and 0.25 kGy. This could be attributed from the results of this study that use of gamma irradiation (Co60) could be beneficial in the control of *Campylobacter* sp. in chilled chicken heart samples.

Though not considered the best indicators of fecal contamination in meat and chicken giblets and also these microorganisms not being part of the food composition, the presence of *Campylobacter* points out flaws in sample handling/processing or poor sanitary hygienic conditions, which allows the proliferation of these and other enteropathogens (Franco, 2012; Franco and Landgraf, 2008). Thus, the contaminated samples analyzed herein are a public health concern.

Keener et al. (2004) have reported that the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) approved the irradiation of poultry meat at a maximum dose of 3.0 kGy to control causative pathogens of foodborne illness, such as *Campylobacter* sp. No microbiological standard regarding the presence of *Campylobacter* sp. exists in the current Brazilian health legislation RDC no. 12 (Brasil, 2001). Thus it is not possible to assume harm from any random value of *Campylobacter* sp. in samples in this regard, and the samples analyzed herein are therefore contaminated, thus unfit for consumption and pose public health risks.

Conclusions

Co_{60} gamma irradiation, when applied to chilled chicken heart at doses of 1.5, 3.0 and 4.5 kGy, was effective in the elimination of *Campylobacter sp*. that was initially present in the samples. Comparisons with the literature indicated that, 1.5 kGy dose would be sufficient in eliminating this microorganism, as it shows sensitivity to low gamma radiation doses.

However, statistically significant differences were not observed among the four groups (control, 1.5kGy, 3.0kGy and 4.5kGy) in any of the analyses carried out. No microbiological standard regarding the presence of *Campylobacter* sp. exists in the current Brazilian health legislation RDC no. 12. Thus, the samples evaluated in the present study were contaminated, therefore unfit for consumption. The presence of these microorganisms in the analyzed samples indicates the need to improve the hygienic-sanitary standards in the production line and preparation of chicken giblets, together with repeated health education for handlers, employees and consumers about the dangers and risks to which they are subjected to.

The presence of high bacterial load in the control samples were observed in the present study. Inspection is therefore imperative with regard to the production, transport and slaughter of poultry. Compliance with regulatory standards and Good Handling Practices also ensure the quality and safety of this type of food and thereby prevents risks to public health.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

Directed at professionals and institutions that contributed to the development of this research:
To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). To Departamento de Engenharia Nuclear da COPPE, Laboratório de Instrumentação Nuclear, Centro de Tecnologia, Universidade Federal do Rio de Janeiro. Especially to CCAMP - Coleção de *Campylobacter*, Laboratório de Zoonoses Bacterianas, IOC/Fundação Oswaldo Cruz/RJ/ Brazil.

REFERENCES

Ahn DU, Kim IS, Lee EJ (2013). Irradiation and additive combinations on the pathogen reduction and quality of poultry meat. Poultry Sci. 92:534-545.

Brazilian Association of Animal Protein (ABPA) (2015). Poultry and Swine Brazil: Production and Export; Forecasts for 2015 and 2016. December 09, 2015. Available from <% 20Brasileira Association% 20of% 20Proteína% 20Animal% 20_% 20ABPA .html>. Acess on January 2016.

Poultry Brazil (2012). Chicken meat, unanimously that runs from north to south of Brazil. Poultry Brazil, UBABEF. 1:8-14.

Azeredo LI (2007). Irradiation of chicken fresh and in vitro livers contaminated with *Campylobacter jejuni*. 2007. 57f. (Monograph Specialization Course Conclusion on Food Irradiation) - Faculty of Veterinary Medicine, Federal Fluminense University. Niteroi. Brazil.

Azeredo LI, Luchese RH, Filgueiras ALL (2010). *Campylobacter* spp in fresh meat poultry: Chilling step evaluation. J. Inst. Adolfo Lutz. Sao Paulo. 69(4):518-524.

Bier O (1980). Bacteriology and Immunology: its applications to medicine and hygiene. Sao Paulo: Ed. Melhoramentos. 1062 p.

Bognar BZ, Silva RC, Ribeiro AB (2012). Contamination by *Campylobacter* spp. in faeces, giblets and meat chicken. SaBios: J. of Health and Biol. 7 (2):16-24.

Brasil (1996). Agriculture Ministry Fisheries and Food Supply. Ordinance no. 304 of 22 April 1996. The cattle slaughter establishments, buffalos and pigs may only deliver meats and giblets, for marketing, with temperatures of up to seven (7) degrees centigrade. Published in the Official Gazette, Brasilia, DF, on 23 April 1996.

Brasil (2001). Health Ministry. National Health Surveillance Agency (NHSA). RDC Resolution no. 12 of 2 January 2001. Approves the Technical Regulation on microbiological standards for food and Annexes I and II. Official Gazette [of] the Federative Republic of Brazil, Brasilia, DF, p. 45-53, section 1, of January 10, 2001.

Campos TM, Mendes GS, Duque SS, Esteves WTC, Thomé JDS, Filgueiras ALL (2015). Transmission of Campylobacter spp. through chicken meat and giblets marketed in the state of Rio de Janeiro, Brazil. J. VISA under Debate. 3 (1):53-60.

Caruso LIA, Souza MCL, Filgueiras ALL, Duque SS, Esteves WTC, Thomé JDS (2011). Searching to combine technologies for safer food attainment. Ciên. Tecnol. Alim. Campinas. 31(2):380-387.

Chun HH, Kim JY, Lee BD, Yu DJ, Song KB (2010). Effect of uv-c irradiation on the inactivation of inoculated pathogens and quality of chicken breasts during storage. Food Control 21:276-80.

Clavero MRS, Monk JD, Beuchat LR, Doyle MP, Brackett RE (1994). Inactivation of *Escherichia coli* O157:H7; *Salmonella e* and *Campylobacter jejuni* in raw ground beef by gamma irradiation. Appl. Environ. Microbiol. 60(6):2069-2075.

Clements M (2011). Campylobacter control during poultry slaughter and processing. Available from: <http://www.wattagnet.com/articles/9118-campylobacter-control-during-poultry-slaughter-and-proces sing>. Acess on Feb 2016.

Diehl JF (1995). Safety of irradiated foods. 2. ed. New York: Marcel Dekker, Inc. P 432.

Filgueiras ALL, Hofer E (1989). Occurrence of thermophilic *Campylobacter* at different points of a sewage treatment plant in the city of Rio de Janeiro, RJ. J. Microbiol. 20:303-308.

Franco BDGM, Landgraf M (2008). Food microbiology. Col. Maria Teresa Destro. São Paulo: Ed Atheneu.. 182 p.

Franco RM (2012). Etiological agents of foodborne diseases. Niterói: Ed. UFF (Didactic Collection). 120 p.

Freitas JA, Noronha GN (2007). Occurrence of *Campylobacter* spp. in chicken meat and giblets exposed to consumption in Belém, Pará. Brazil. Arch. of Vet. Med. and An. Sci. 59(3):813-815.

Gava AJ (2008). Food technology: Principles and applications. Sao Paulo: Ed. Nobel. 512 p.

Haughton P, Grau EG, Lyng J, Cronin D, Fanning S, Whyte P (2012). Susceptibility of *Campylobacter* to high intensity near ultraviolet/visible 395 ± 5 nm light and its effectiveness for the decontamination of raw chicken andcontact surfaces. Int. J. Food Microbiol. 159:267-273.

Hernandes NK, Vital HC, Sabaa-Srur AUO (2003). Food irradiation: advantages and limitations. Bull. Brazil. Soc. Food Sci. Technol. Campinas. 37(2):154-159.

Keener KM, Bashor MP, Curtis PA, Sheldon BW, Kathariou S (2004). Comprehensive review of *Campylobacter* and poultry processing. Compr. Rev. Food Sci. Food Safety 3:105-16.

Kudra LL, Sebranek JG, Dickson JS, Mendonca AF, Zhang Q, Jackson-Davis A, Prusa KJ (2012). Control of *Campylobacter jejuni* in chicken breast meat by irradiation combined with modified atmosphere packaging including carbon monoxide. J. Food Protect. 75(10):1728-1733.

Lior H (1982). A new, extend biotyping sheme for *Campylobacter jejuni*, Campylobacter coli and Campylobacter laridis. J. Clin. Microbiol. 20:636-640.

Mendonça AF (2002). Inactivation by irradiation. In: Juneja V and Sofos J. Control of Foodborne Microorganisms. ed. Marcel Dekker Inc., New York, NY.

Miranda ZB (2012). Food irradiation: myth or reality? An. Busin. Brazil. 2: 56-65, National Society of Agriculture Available from <https://issuu.com/socie dadenacionaldeagricultura / docs / abb_02>. Acess on may 2012.

Olson DG (1998). Irradiation of food. Food Technol. 52:56-62.

Patterson MF (1995). Sensitivity of *Campylobacter* spp. to irradiation in poultry meat. Lett. Appl. Microbiol. 20(6):338-40.

Raut AD, Shashidhar R, Bandekar JR, Kapadnis RB (2012). Effectiveness of radiation processing in elimination of *Campylobacter* from poultry meat. Radiat. Physics Chem. 81:82-85.

Russell SM (2009). How do campylobacter end up on chicken? Available at: http://www.wattagnet.com/articles/296-how-do-campylobacter-end-up-on-chic ken.

Trassi AMC (2012). Occurrence of Campylobacter spp. in chicken meat. Goiânia. 2012. 45f. Dissertation (Masters in Animal Science) - School of Veterinary and Animal Science, Federal University of Goiás, Goiânia / GO.

academicJournals

Vol. 10(40), pp. 1720-1727, 28 October, 2016
DOI: 10.5897/AJMR2016.8201
Article Number: EE48C5661350
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Soil enzyme activities and soil microbe population as influenced by long-term fertilizer management during the barley growth in Hunan Province, China

Haiming Tang*, Xiaoping Xiao, Jimin Sun , Lijun Guo, Ke Wang, Weiyan Li and Wenguang Tang

Hunan Soil and Fertilizer Institute, Hunan, Changsha, 410125, China.

Received 8 June 2016 Accepted 27 September, 2016

The effects of long-term fertilizer management on soil enzyme activities and soil microbe population under double-cropping paddy fields in southern China was studied. The purpose of this study was to explore the changes of soil enzyme activities and soil microbe population as related to mineral fertilizer and manure and rice residue based on a long-term field experiment. The experiment was initiated in 1986 and consisted of five treatments: without fertilizer (CK), mineral fertilizer (MF), rice residue plus mineral fertilizer (RF), low manure rate plus mineral fertilizer (LOM), and high manure rate plus mineral fertilizer (HOM). The cropping system consisted of barley (*Hordeum vulgare* L.), early rice (*Oryza sativa* L.) and late rice. In 2013-2014, soil samples were collected from the 0-20 cm layers to determine soil enzyme activities and soil microbe abundance during barley growth phases. The results indicated that during the barley growing season, the enzyme activities were higher in the HOM, LOM and RF than in the CK. The treatments of HOM, LOM and RF also improved the numbers of aerobic bacteria, actinomycetes and fungi. During barley growth phases, combined application of manure, crop residue and chemical fertilizer improved soil enzyme activities and soil microbe population.

Key words: Alkaline phosphatase, arylamidase, β-glucosidase, *Hordeum vulgare* L., manure, microbial abundance, mineral fertilizer, rice residue.

INTRODUCTION

Soil microbes play an important role in the ecosystem functioning and are important in maintaining the soil fertility, nutrient cycling and organic matter decomposition (Pastor et al., 1984), which are dependent on the composition of soil microbial communities (Robertson et al., 2000; Singh et al., 2010), and relative to microbial diversity, community structure. Soil enzymes play important role in energy transfer and they are very important for soil quality and crop growth, which are used as indices for soil microbial activity and fertility (Benitez et al., 2000; Dick, 1994; Tabatabai, 1994). Soil enzymes respond to soil practice changes more quickly than other soil factors, therefore it was used as early indicators of biological changes. The soil enzyme reflects soil

*Corresponding author. E-mail: tanghaiming66@163.com.

Table 1. The fertilizer management information under different fertilizer treatments (kg ha).

Treatment	Barley			Total		
	N	P	K	N	P	K
MF	157.5+0*	43.2+0	81.0+0	157.5	43.2	81.0
RF	133.0+24.5	37.8+5.4	48.2+32.8	157.5	43.2	81.0
LOM	110.2+47.3	21.8+21.4	51.1+29.9	157.5	43.2	81.0
HOM	63.0+94.5	0.5+42.7	21.2+59.8	157.5	43.2	81.0
CK	0+0	0+0	0+0	0	0	0

* Input from mineral fertilizer + input from organic fertilizer. CK: without fertilizer, MF: mineral fertilizer, RF: crop residue and mineral fertilizer, LOM: low manure rate and mineral fertilizer, HOM: high manure rate and mineral fertilizer. 1) For the RF treatment, rice straw return rate (air dry) was 3600 kg ha. 2) For the LOM treatment, chicken manure (decayed) application rate was 2670 kg ha. 3) For the HOM treatment, chicken manure application rate (decomposed) was 5340.0 kg hm^{-2}. 4) The N, P, and K content of air-dry rice straw was 0.68, 0.15 and 0.91%, respectively, and N, P, and K content of chicken manure (decayed) was 1.77, 0.80 and 1.12%, respectively.

functional diversity, which was affected by environmental conditions and ecological factors, such as soil microorganisms, plants and animals (Nannipieri et al., 2004). Soil enzymes include arylamidase, alkaline phosphatase, β-glucosidase and arylsulfatase, which were important for transformation of C, N, P and S (Tabatabai, 1994).

Recently, some studies have shown that the soil enzymes and microbial activities were affected by field management, such as soil tillage and crop residue management (Ekenler and Tabatabai, 2003; Wu et al., 2004), application of fertilizer and organic matter (Carmine et al., 2004; Tejada et al., 2006; García-Gil et al., 2000), crop rotations (Hamido and Kpomblekou, 2009; Bandick and Dick, 1999), pH, ionic strength and natural organic matter (Kyriakopoulos et al., 2006). However, the effects of long-term fertilizer management on soil enzyme activities and soil microbial abundance under double-cropping paddy fields in southern China should be studied.

In recently years, the traditional fertilization practices have been changing in China's major rice production regions. However, the studies on the effects of long-term fertilizer management on soil enzyme activities and soil microbe population under double-cropping paddy fields in southern China were less. Therefore, the purpose of this study was to explore the changes of soil enzyme activities and soil microbe abundance in a double-cropping rice system as related to the application of manure, crop residue plus mineral fertilizer, and mineral fertilizer based on a long-term field experiment.

MATERIALS AND METHODS

Sites and cropping system

The experiment was started in October 20, 1986, at Ning Xiang County (28°07' N, 112°18' E, and altitude 36 m) of Hunan Province, China. Under a continent monsoon climate, the annual mean precipitation is 1553 mm and potential evapotranspiration is 1354 mm. The monthly mean temperature is 17.2°C. Soil texture of the plough layer (0–20 cm) is silt clay loam with 13.71 sand and 57.73% silt. At the beginning of the study, the characteristics of the surface soil (0–20 cm) are as follows: soil organic carbon (SOC) 29.4 g kg^{-1}, total nitrogen 2.0 g N kg^{-1}, available N 144.1 mg kg^{-1}, total phosphorous 0.59 g P kg^{-1}, available P 12.87 mg kg^{-1}, total potassium 20.6 g K kg^{-1}, and available potassium 33.0 mg kg^{-1}. There are three crops in a year, barley (*Hordeum vulgare* L.), early rice (*Oryza sativa* L.), and late rice. Barley is sown in the middle of November and is harvested in early May of the following year. Early rice is then transplanted, and harvested in the middle of July. The growing season of late rice lasts from late July to the end of October.

Experimental design

There were five treatments: control (without fertilizer, CK), mineral fertilizer (MF), rice residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM). The experiment ensured all fertilized treatments received equal N rate (the amount of N in mineral fertilizer plus that from rice residue or manure). The fertilizer management information are listed in Table 1. Barley is sown in November 15, 2013, and is harvested in May 7, 2014. Early rice is transplanted in May 9, and harvested in July 24. Late rice was transplanted in July 26, and harvested in October 17. Before barley sowing, manure and air–dried rice residue were incorporated into soil surface. Before barley sowing and rice transplanting, the soil were taken tillage, and the cultivation depth was about 20 cm. For barley, 70% of mineral N fertilizer was applied at seeding, and the 30% of N fertilizer was applied at top dressing. The phosphorus and K fertilizers were applied at seeding. The seeding rate application was 250.0 kg ha with each treatment. There were three replications and each plot size was 66.7 m^2 (6.67 × 10 m). We referred to the data for the individual cropping periods as 2013–2014, after 27 consecutive years of position experiment.

Soil sampling and measurements

Data were collected from October 2013 to May 2014. In each plot, soil samples in the ploughed layer (0–20 cm) were collected from

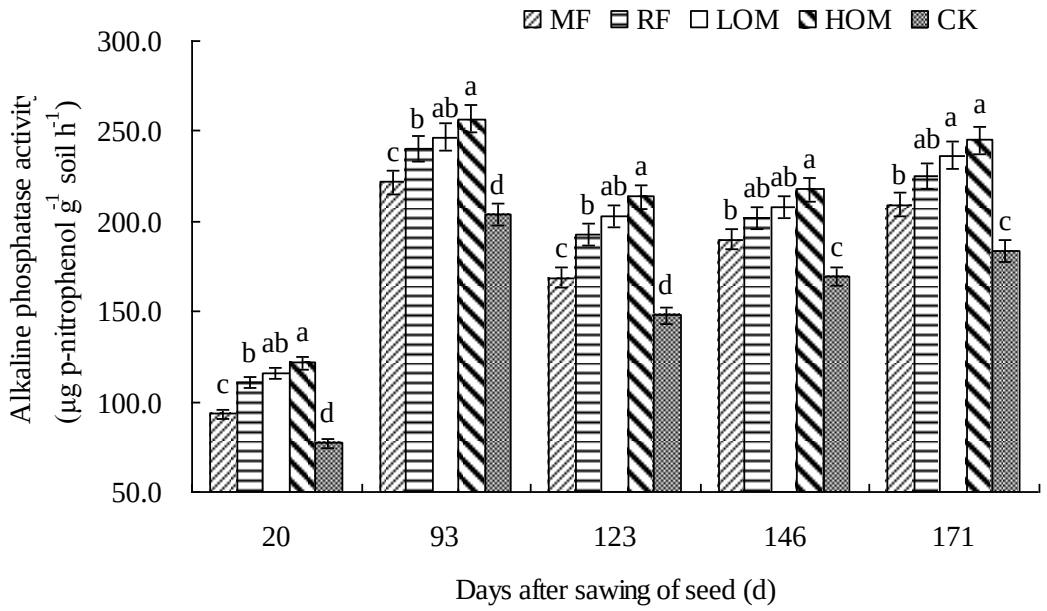

Figure 1. Dynamics of alkaline phosphatase activity in field during barley growth stages, in response to N treatments. MF: mineral fertilizer; RF: rice residues and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. SS: seedling stage; TS: tillering stage; JS: jointing stage; HS: heading stage; MS: maturity stage. Error bars represent the standard error of mean. Different letters indicate significance at $P < 0.05$, according to the least significant difference test.

the centre of four hills of barley plants by using a drill at different barley growth stages, such as the seedling stage, tillering stage, jointing stage, heading stage and maturity stage. Three subsamples were collected from each plot.

The soil samples were passed through a 2-mm sieve and kept moist in a refrigerator at 4°C until analysis. Arylamidase (EC 3.4.11.2) activity was assayed by incubating 1.0 g moist soil with 3.0 mL of 0.1 M THAM buffer (pH 8.0) and 1.0 mL of an 8.0 mM solution of L–leucine β–naphthylamide hydrochloride (Acosta–Martínez and Tabatabai, 2000). Alkaline phosphatase (EC 3.1.3.1), β–glucosidase (EC 3.2.1.21) and arylsulfatase (EC 3.1.6.1) activities were determined as described by Tabatabai (1994), and the activity was reported as μg *p*–nitrophenol g^{-1} h^{-1}. All measurements in the laboratory were repeated for three times. Meanwhile, colony forming units (CFUs) of soil aerobic bacteria, actinomycetes and fungi were enumerated by Wu et al. (2004). Colony forming units (CFUs) of soil aerobic bacteria were enumerated by a 10-fold dilution plate technique. And the number of aerobic bacteria was identified by spreading 100 μl of diluted sample on LB agar medium. Three replicates of the inoculated agar plates were incubated at 28°C for 3 days for bacteria, after which colonies were counted.

Statistical analysis

All data were expressed as mean ± standard error. The data of different treatments at the same growth stage were analyzed as a randomized complete block, using the ANOVA procedure of SAS (SAS Institute, 2003). Mean values were compared using the least significant difference (LSD) test, and a probability value of 0.05 was considered to indicate statistical significance.

RESULTS

Dynamics of alkaline phosphatase activity during barley growth stages

Under different fertilization treatments during the barley growth, alkaline phosphatase activity in the MF, RF, LOM and HOM soils was higher than that in the control plot (Figure 1). In the barley growing season, the order of alkaline phosphatase activity was: HOM>LOM>RF> MF>CK, and there were significant differences (P <0.05) between HOM, LOM, RF, MF and CK. In the barley growing season, the alkaline phosphatase activity under different N treatments was in the range of 93.07–221.79, 110.41–240.13, 115.60–246.85, 121.53–256.77 and 76.92–203.64 μg *p*-nitrophenol g^{-1} soil h^{-1}, respectively. The highest activity was detected at the tillering stage (TS) (Figure 1).

Dynamics of β-glucosidase activity during barley growth stages

During barley growing stages, the activity of soil β-glucosidase was significantly affected by manure, rice residue and mineral fertilizer additions. The highest β-glucosidase activity was observed with HOM and the

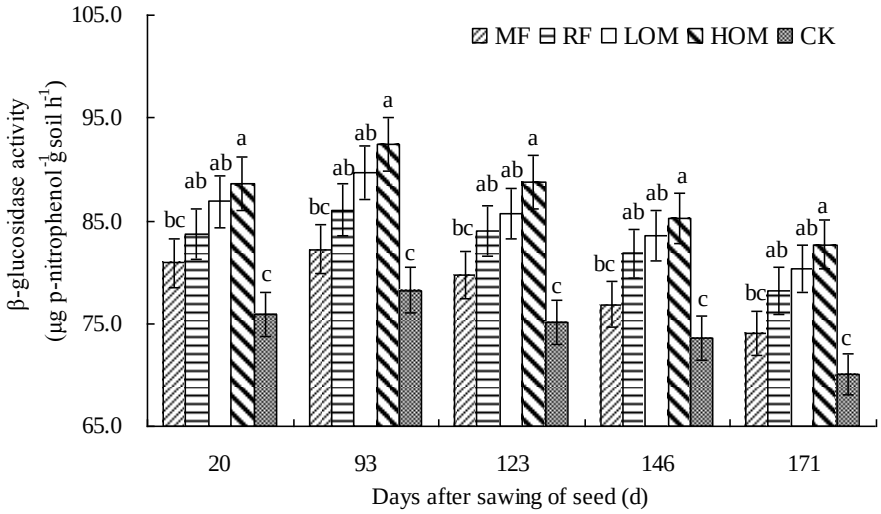

Figure 2. Dynamics of β-glucosidase activity in field during barley growth stages, in response to N treatments. MF: mineral fertilizer; RF: rice residues and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer.
SS: seedling stage; TS: tillering stage; JS: jointing stage; HS: heading stage; MS: maturity stage. Error bars represent the standard error of mean. Different letters indicate significance at $P < 0.05$, according to the least significant difference test.

lowest activity with CK (Figure 2), and the order of β-glucosidase activities was as follows: HOM>LOM> RF>MF>CK. In the barley growing season, there was a significant difference ($P < 0.05$) in β-glucosidase activity under the HOM and MF treatment at the seedling stage (SS), TS, jointing stage (JS), heading stage (HS) and maturity stage (MS).

Dynamics of arylsulfatase activity during barley growth stages

During barley main growing stages, soil arylsulfatase activities with different treatments was in the range of 26.45–32.56, 29.72–34.30, 30.05–35.68, 31.52–36.97 and 24.10–31.45 μg *p*–nitrophenol g^{-1} soil h^{-1}, respectively (Figure 3). There was no significant difference ($P > 0.05$) in arylsulfatase activities between the MF and CK, but the arylsulfatase activities in HOM and LOM was significantly ($P < 0.05$) higher than that in MF, CK at the main growth stages of barley.

Dynamics of arylamidase activity during barley growth stages

The application of the manure, rice straw and mineral fertilizers significantly affected soil arylamidase activity (Figure 4). At 0–20 cm soil depth, there was no significant difference ($P > 0.05$) in soil arylamidase activity under the HOM, LOM and RF, but these activities were significantly ($P < 0.05$) higher than that in CK in all growth stages of barley. In the barley growing season, arylamidase activity changed in the range of 22.75–35.54, 24.97–37.67, 25.62–38.95, 26.47–39.88 and 20.42–32.01 μg *p*-nitrophenol g^{-1} soil h^{-1}, respectively.

Enumeration of aerobic bacteria, fungi and actinomycetes in soil

During barley growth stage, there were significant differences ($P < 0.05$) in the numbers of aerobic bacteria, fungi and actinomycetes between HOM, LOM, RF, MF and CK. The order of number of aerobic bacteria was RF>MF>HOM=LOM>CK at the barley main growing stages (Table 2). Also, the numbers of soil fungi and actinomycetes with manure, rice straw and mineral fertilizer were significantly higher ($P < 0.05$) than the control at different barley stages. And the number of soil actinomycetes and fungi decreased in the order HOM>LOM>RF>MF>CK in the barley growing season.

DISCUSSION

Soil enzyme activities under different fertilizer N management during barley growth

Soil enzymes responded to management practices more

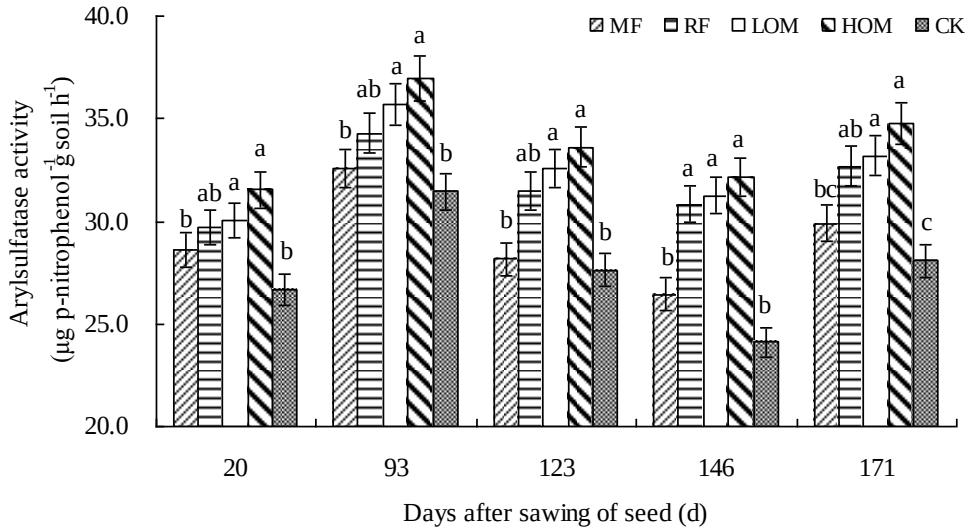

Figure 3. Dynamics of arylsulfatase activity in the field during barley growth stages, in response to N treatments. MF: mineral fertilizer; RF: rice residues and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. SS: seedling stage; TS: tillering stage; JS: jointing stage; HS: heading stage; MS: maturity stage. Error bars represent the standard error of mean. Different letters indicate significance at $P < 0.05$, according to the least significant difference test.

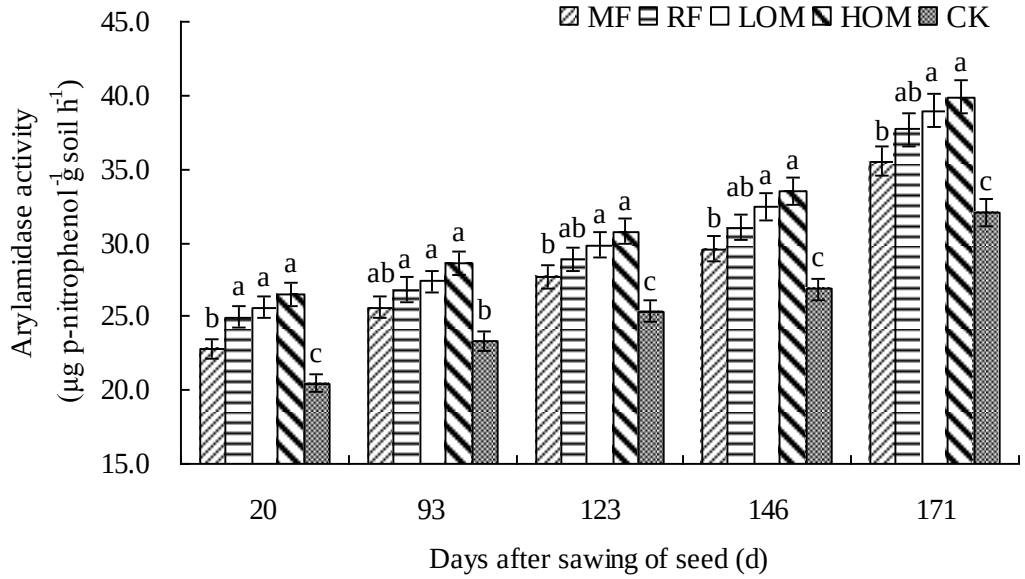

Figure 4. Dynamics of arylamidase activity in the field during barley growth stages, in response to N treatments. MF: mineral fertilizer; RF: rice residues and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. SS: seedling stage; TS: tillering stage; JS: jointing stage; HS: heading stage; MS: maturity stage. Error bars represent the standard error of mean. Different letters indicate significance at $P < 0.05$, according to the least significant difference test.

quickly than other soil factors, therefore, it was used as early indicator of biological changes. Wu et al. (2014) showed that the activity of soil enzymes was positively correlated with concentrations of soil available phosphorus and available nitrogen in continuous cropping soil. At the barley main growth stages, there were

Table 2. Variations of (cfu g^{-1} dry soil) soil aerobic bacteria, fungi and actinomycetes at different barley growth stages and under different fertilization treatments.

Items	Treatment	Barley growth stage				
		SS	TS	JS	HS	MS
Aerobic bacterial (×10^4cfu g^{-1} dry soil)	MF	249.03±8.17^b	59.81±6.16^d	110.11±7.61^d	120.46±7.89^d	103.05±8.18^d
	RF	282.92±7.19^a	106.11±4.25^c	156.41±5.70^c	166.76±5.98^c	140.87±6.27^c
	LOM	159.86±4.61^c	147.26±3.06^b	197.56±4.52^b	206.98±4.81^b	217.33±4.07^b
	HOM	156.64±4.52^c	213.44±1.73^a	263.74±3.18^a	273.16±3.48^a	283.46±2.97^a
	CK	117.18±3.38^d	49.59±1.43^d	99.89±2.88^d	109.31±3.16^d	81.49±2.35^e
Fungi (×10^2 cfu g^{-1} dry soil)	MF	37.71±2.34^c	44.46±2.54^d	56.33±3.71^c	50.49±3.47^d	47.25±3.37^d
	RF	61.46±2.23^b	69.18±2.45^c	97.03±3.01^b	93.16±2.97^c	90.78±2.91^c
	LOM	77.32±1.77^a	85.04±1.99^b	104.05±2.80^b	102.75±2.69^b	100.95±2.62^b
	HOM	81.03±1.09^a	88.06±1.28^a	128.48±1.63^a	120.36±1.46^a	116.84±1.36^a
	CK	27.17±0.78^d	33.71±0.97^e	38.58±1.11^d	37.59±1.09^e	35.86±1.04^e
Actinomycetes (×10^3 cfu g^{-1} dry soil)	MF	105.09±5.54^d	84.85±4.95^d	119.33±6.05^c	90.71±5.80^c	98.66±5.41^d
	RF	158.11±4.98^c	137.87±4.39^c	139.26±5.78^b	130.64±5.53^b	142.17±4.50^c
	LOM	172.41±4.56^b	152.17±3.98^b	200.31±4.02^a	191.69±3.77^a	155.79±4.10^b
	HOM	191.84±3.03^a	171.60±2.45^a	209.65±3.44^a	201.03±2.62^a	187.30±2.85^a
	CK	84.06±2.43^e	63.82±1.84^e	67.11±1.94^d	50.49±1.46^d	58.68±1.69^e

MF: mineral fertilizer; RF: rice residues and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. SS: seedling stage; TS: tillering stage; JS: jointing stage; HS: heading stage; MS: maturity stage. Values are presented as mean ± SE (n = 3). Different lowcase letters indicate significance at $P < 0.05$, according to the least significant difference test.

significant differences ($P > 0.05$) in alkaline phosphatase activity between HOM, LOM, RF, MF and CK treatments. The results showed that alkaline phosphatase activity were affected by application of manure and residue management practices. Temporal variations in activity, induced by manure and crop residue decomposition, may promote the enzymes by the microbial biomass. Increase of alkaline phosphatase activity in HOM, LOM, RF and MF treatments suggested that there was a stimulus of P-supplying mechanisms to the microbial community with fertilizer or manure, crop-derived inputs (Ritz et al., 1997). And there were significant differences ($P > 0.05$) in alkaline phosphatase activity under the HOM and MF treatments at the main growth stages of barley. And the difference in the enzyme activity was in response to mineral fertilizer, manure, and straw incorporation or crop rotation practices used in this study.

β-Glucosidase is the enzyme for degradation of organic compounds in soil which plays a crucial role in the C cycle in soils. β-Glucosidase releases important energy sources for microorganisms. Bandick and Dick (1999) and Ekenler and Tabatabai (2003) showed that the activity of β-glucosidase was affected by different residue management practices. These studies showed that there are significant difference in β-glucosidase activity among the mineral fertilizer, rice residue and mineral fertilizer, manure and mineral fertilizer and without fertilizer treatments at the barley main growth stages. There are close relationship between glucosidases and β-glucosidase activity, which glucosidases hydrolyzed to glucose. The increase of β-glucosidase activity and glucosidases with HOM, LOM and RF treatments was possibly related to the increased mineralization of organic matter added with the manure and rice residue, which may provide the higher C substrates for β-glucosidase and glucosidases activity.

Arylsulfatase is an extracellular enzyme which plays an important role in sulfur (S) recycle for soil and plant (Tejada et al., 2006). This study showed that the soil activity of arylsulfatase with HOM, LOM, RF treatments were higher than that of the MF, CK treatments at the barley main growth stages. These observations indicated that manure, rice residue and mineral fertilizer made higher contribution to the SOM than without fertilizer, which may explain the higher immobilization in rhizosphere soil containing manure, rice residue and mineral fertilizer and fertilizer than in rhizosphere soil containing without fertilizer. In the present study, differences in soil arylsulfatase activity were related to the quantity of substrate contained in the organic and rice straw incorporation.

Soil arylamidase plays an important role in N mineralization in soils (Acosta-Martínez and Tabatabai, 2000; Castellano and Dick, 1988). Previous studies demonstrated that the arylamidase activity was affected by residue management (Deng and Tabatabai, 1997). In this study, statistical analyses suggested that arylamidase activity was affected by different fertilizer management

(Figure 4). Dick et al. (1988) reported that the arylamidase activity decreased with long-term addition of inorganic N, whereas the activity increased with crop residues additions in wheat-fallow system. The low activity of arylamidase at the main growth stages of barley might be related to the fertilizer management. The reason maybe the difference in decomposable organic material in the manure, crops straw-returned soil which favored soil enzyme activity (Luo et al., 2011; Sharma and Arora, 2011).

Soil microbial abundance in response to fertilizer N management

Enami et al. (2001) suggested that soil microbial community structure was affected by rice straw. Kyriakopoulos et al. (2006) also showed that soil microbial community structure was affected by pH, ionic strength and natural organic matter. It was discovered that the numbers of soil aerobic bacteria, actinomycetes and fungi increased by application of manure and rice residue. In the present study, as compared to the without manure soil, the numbers of soil aerobic anaerobic microorganisms were increased with manure and rice straws treatments (Table 2). This might be due the difference of decay rates in the manure and crops straws-returned soil which favored aerobic bacteria. As a result, the numbers of aerobic bacteria with HOM, LOM, RF treatments were increased at barley main growth stages. Some studies indicated that soil fungi are important for the formation and stabilization of soil aggregates; however, soil fungi are also sensitive to environmental change (David et al., 2012; Li et al., 2012). This research indicated that the number of soil fungi decreased following HOM>LOM>RF>MF>CK at barley main growth stages. The reason may be that the changes of soil environmental on fungal diversity could influence ecosystem function via decomposition of manure and crops straws, so the soil fungi populations were increased by application with manure and rice straws at barley main growth stages. Present results also indicate an increase of actinomycetes number with manure and rice residue application at barley main growth stages. And the differences in the different treatments may have resulted in the differences in the numbers of aerobic bacteria, actinomycetes and fungi between the applied with manure, rice straws and without organic input treatments. And the further studies could be helpful to better understand these changes in the number of soil microbial impact on the soil microbial functions and nutrient availability.

Conclusion

Soil microbial abundance and enzyme activities are the main driving factors for organic matter decomposition and play an important role in soil nutrient transformation. This study indicated that soil enzyme activities and soil microbial abundance were affected by the application of manure, crop residue and mineral fertilizer practices during barley growth. Combined application of manure, crop residue and mineral fertilizer stimulated soil β-glucosidase, alkaline phosphatase, arylsulfatase and arylamidase activities. The numbers of soil microbes also increased when mineral and organic fertilizers were added. The application of mineral fertilizer was beneficial for microbial abundance, but the enhancement was lower than that of HOM, LOM and RF treatments. Therefore, these findings indicated that soil enzyme activity and soil microbe population were improved by using HOM, LOM and RF treatments. As a result, it is the best way to maintain soil fertility by using HOM, LOM and RF fertilization models.

Conflict of Interest

There is no conflict of interest

ACKNOWLEDGEMENT

This study was supported by the National Natural Science Foundation of China (No. 31201178, 31571591).

REFERENCES

Acosta–Martínez V, Tabatabai MA (2000). Arylamidase activity of soils. Soil Sci. Soc. Am. J. 64:215-221.

Bandick AK, Dick RP (1999). Field management effects on soil enzyme activities. Soil Biol. Biochem. 31:1471-1479.

Benitez ER, Melgar H, Sainz M, Nogales R (2000). Enzyme activities in the rhizosphere of pepper (*Capsicum annuum* L.) grown with olive cake mulches. Soil Biol. Biochem. 32:1829-1835.

Carmine CC, Magda DRP, Maria R, Pacifico R (2004). Effects of municipal solid waste compost amendments on soil enzyme activities and bacterial genetic diversity. Soil Biol. Biochem. 36:1595-1605.

Castellano SD, Dick RP (1988). Distribution of sulfur fractions in soil as influenced by management of organic residues. Soil Sci. Soc. Am. J. 52:1403-1407.

David JB, Kurt AS, Juan CL, Jared LD (2012). Soil fungi influence the distribution of microbial functional groups that mediate forest greenhouse gas emissions. Soil Biol. Biochem.53: 112–119.

Deng SP, Tabatabai MA (1997). Effect of tillage and residue management on enzyme activities in soils: III. Phosphatases and arylsulfatase. Biol. Fert. Soil 24: 141–146.

Dick RP (1994). Soil Enzyme Activities as Indicators of Soil Quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (Eds.). Defining soil quality for a sustainable environment. American Society of Agronomy, Madison, WI, Pp.107-124.

Dick RP, Rasmussen PE, Kerle EA (1988). Influence of long-term residue management on soil enzyme activity in relation to soil chemical properties of a wheat-fallow system. Biol. Fert. Soil 6:159-164.

Ekenler M, Tabatabai MA (2003). Tillage and residue management effects on β–glucosamidase activity in soils. Soil Biol. Biochem.35:871-874.

Enami Y, Okano S, Yada H, Nakamura Y (2001). Influence of earthworm activity and rice straw application on the soil microbial community structure analyzed by PLFA pattern. Eur. J. Soil Biol. 37:269-272.

García-Gil JC, Plaza C, Soler-Rovira P, Polo A (2000). Long-term effects of municipal solid waste compost application on soil enzyme activities and microbial biomass. Soil Biol. Biochem. 32:1907-1913.

Hamido SA, Kpomblekou AK (2009). Cover crop and tillage effects on soil enzyme activities following tomato. Soil Till. Res. 105:269-274.

Kyriakopoulos G, Doulia D, Hourdakis A (2006). Effect of ionic strength and pH on the adsorption of selected herbicides on amberlite. Int. J. Environ. Anal. Chem. 86(3&4):207-214.

Li YJ, Chen X, Shamsi IH, Fang P, Lin XY (2012). Effects of irrigation patterns and nitrogen fertilization on rice yield and microbial community structure in paddy soil. Pedosphere 22(5):661-672.

Luo YJ, Wang ZF, Gao M, Wei CF (2011). Effects of conservation tillage on organic carbon, nitrogen and enzyme activities in a hydragric anthrosol of Chongqing, China. Energy Procedia 5:30-36.

Nannipieri P, Kandeler E, Ruggiero P (2004). Enzyme Activities and Microbiological and Biochemical Processes in Soil. In: Burns RG, Dick RP (Eds.). Enzymes in the environment. Activity, Ecology and Applications. Dekker, New York, pp.1-33.

Pastor J, Aber JD, McClaugherty CA, Melillo JM (1984). Above–ground production and N and P cycling along a nitrogen mineralization gradient on Blackhawk Island, Wisconsin. Ecol. 65:256-268.

Ritz K, Wheatley RE, Griffiths BS (1997). Effects of animal manure application and crop plants upon size and activity of soil microbial biomass under organically grown spring barley. Biol. Fert. Soil 24: 372-377.

Robertson GP, Paul EA, Harwood RR (2000). Greenhouse gases in intensive agriculture: contributions of individual gases to the radiative forcing of the atmosphere. Science 289:1922-1925.

SAS Institute (2003). SAS Version 9.1.2 2002–2003. SAS Institute Inc., Cary, NC.

Sharma RK, Arora DS (2011). Solid state degradation of paddy straw by *Phlebia floridensis* in the presence of different supplements for improving its nutritive status. Int. Biodeter. Biodegr. 65:990-996.

Singh BK, Bardgett RD, Smith P, Reay DS (2010). Microorganisms and climate change: terrestrial feedbacks and mitigation options. Nat. Rev. Microbiol. 8:779-790.

Tabatabai MA (1994). Soil Enzymes. In: Page AL, Miller RH, Keeney DR (Eds.), Methods of soil analysis. American Society of Agronomy, Madison, pp. 775-833.

Tejada M, Garcia C, Gonzalez JL, Hernandez MT (2006). Use of organic amendment as a strategy for saline soil remediation: Influence on the physical, chemical and biological properties of soil. Soil Biol. Biochem. 38:1413-1421.

Wu JX, Dong YH, Li JG, Jiang JF (2014). Influences of different fertilizing strategies of continuous cropping on lettuce yield, soil nutrients and enzymatic activities in greenhouse cultivation. J. Food Agric. Environ. 12(2):661-665.

Wu WX, Ye QF, Min H, Duan XJ, Jin WM (2004). Bt–transgenic rice straw affects the culturable microbiota and dehydrogenase and phosphatase activities in a flooded paddy soil. Soil Biol. Biochem. 36:289-295.


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