PHENOTYPIC CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITIES OF *EWINGELLA AMERICANA* AND *KLUYVERA INTERMEDIA* ISOLATED FROM SOAKED HIDES AND SKINS

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Soaked hides and skins may contain different species of family Enterobacteriaceae, originating from animal's feces, soil, and water. Some species of this family may be pathogenic to humans and animals. Hence, phenotypic characteristics and antibiotic susceptibilities of Ewingella americana and Kluyvera intermedia belonging to Enterobacteriaceae were explained in this study. While Ewingella americana was isolated from only one soaked hide, Kluyvera intermedia was isolated from both soaked hide and skin. Phenotypic characterization of these isolates was performed using API 20E test kit. Antibiotic susceptibilities of these isolates were examined by Kirby Bauer Disc Diffusion Test using piperacillin/tazobactam (110µg), amoxicillin/clavulanate (30µg), ampicillin/sulbactam (20µg), amikacin (30µg), tobramycin (10µg), kanamycin (30µg), gentamicin (10µg), streptomycin (10µg), ampicillin (10µg), imipenem (10µg), meropenem (10µg), cefoxitin (30µg), cefuroxime sodium (30µg), ceftazidime (30µg), cephalothin (30µg), ceftriaxone (30µg), norfloxacin (10µg), nalidixic acid (30µg), ofloxacin (5µg), ciprofloxacin (10µg), sulfamethoxazole-trimethoprim (25µg), aztreonam (30µg), chloramphenicol (30µg), tetracyline (30µg). Ewingella americana was resistant against aztreonam, ceftazidime, ceftriaxone but this isolate was intermediate susceptible against cefuroxime sodium, ampicillin, nalidixic acid, tetracyline and chloramphenicol. Kluyvera intermedia was found to be resistant against streptomycin, cephalothin, aztreonam, and ampicillin but it was intermediate susceptible to amikacin, kanamycin, chloramphenicol, imipenem, ceftazidime, ceftriaxone, amoxicillin/clavulanate and ciprofloxacin. Moreover, both isolates were found to be susceptible to other antibiotics. Therefore, effective antibacterial applications should be applied to kill these antibiotic resistant bacteria.

Keywords: Ewingella americana, Kluyvera intermedia, antibiotics, soaked hides and skins

INTRODUCTION

Freshly slaughtered hides/skins may have different species of microorganisms which are related to normal bacterial flora of animal's skin and feces, soil, water and air (Birbir and Ilgaz, 1996; Aslan and Birbir, 2012; Birbir *et al*., 2016). To prevent bacterial growth and decomposition of hides/skins, moisture contents of these organic materials are reduced by drying, salt or brine curing until the beamhouse processes(Bailey, 2003). However, if the curing process has not performed adequately, salt tolerant bacteria, halophilic microorganisms and bacterial members of family *Enterobacteriaceae* may grow and damage to cured and soaked hides/skins (Bailey and Birbir, 1993; Bailey and Birbir, 1996; Birbir and Ilgaz, 1996; Berber and Birbir, 2010; Aslan and Birbir, 2012; Ulusoy and Birbir, 2015; Akpolat *et al*., 2015; Caglayan *et al*., 2017).

In soaking, the hides/skins are rehydrated and dirt, salt, blood, urine, manure, and interfibrillary material have been removed from the hides/skins. Long process duration, high organic content of soaking solution support growth and bacterial activities. Hence, antimicrobial agent is added into soaking solution but high organic content of soak liquor may adversely affect the efficiency of antimicrobial agent. In the study of Rangarajan *et al*. (2003), species of *Bacillus*, *Chromobacter*, *Pseudomonas*, *Clostridium*, *Lactobacillus* and *Serratia* were isolated from soak liquor. It was reported that bacterial populations in first soaking process decreased rapidly but tended to increase over time.

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In another study, Pfleiderer *et al*. (1988) isolated bacterial species belonging to genera *Proteus*, *Pseudomonas*, *Bacillus*, *Corynebacterium*, *Clostridium*, *Chromobacter*, *Lactobacillus*, *Micrococcus*, *Sarcina*, *Staphylococcus* and *Serratia* from soak liquors. Moreover, bacterial species of genera *Citrobacter, Edwardsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Proteus, Salmonella, Serratia* and *Yersinia* belonging to family *Enterobacteriaceae* were isolated from ten salted cattle hides (Aslan and Birbir, 2012). Ulusoy and Birbir (2015) stated that total numbers of *Enterobacteriaceae*; total numbers of proteolytic *Enterobacteriaceae*; total numbers of lipolytic *Enterobacteriaceae* on salted hides and skins were found as between $1.7x10^4$ cfu/g-$4.5x10^5$ cfu/g and between $1.7x10^5$ cfu/g-$1.5x10^6$ cfu/g; between $9.1x10^3$ cfu/g-$3.9x10^5$ cfu/g and between $1.2x10^5$ cfu/g-$1.1x10^6$ cfu/g; between $6.0x10^2$ cfu/g-$3.7x10^5$ cfu/g and between $1.7x10^4$ cfu/g-$5.0x10^5$ cfu/g, respectively.

The family *Enterobacteriaceae* contains 56 genera (*Arsenophonus*, *Biostraticola*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Dickeya*, *Calymmatobacterium*, *Cedecea*, *Citrobacter*, *Cosenzaea*, *Cronobacter*, *Sodalis*, *Pragia*, *Edwardsiella*, *Enterobacillus, Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Franconibacter, Gibbsiella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Levinea*, *Lonsdalea*, *Mangrovibacter*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Serratia*, *Pantoea*, *Pectobacterium*, *Phaseolibacter*, *Photorhabdus*, *Plesiomonas*, *Proteus*, *Providencia*, *Raoultella*, *Rouxiella*, *Saccharobacter*, *Salmonella*, *Samsonia*, *Shigella*, *Shimwellia*, *Tatumella*, *Thorsellia*, *Rahnella, Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia, Yokenella*) 454 named species (http://www.bacterio.net/-classifphyla.html#bacteria). All members of the family *Enterobacteriaceae* are facultative anaerobic, chemoorganotrophic, non-halophilic, non-sporulating, rod-shaped Gram-negative bacteria. Although most of the enteric bacteria may be motile by peritrichous flagella, a few of them are nonmotile. *Enterobacteriaceae* may be found in water, soil, animals and plants. Some species may be pathogenic to humans, animals, plants and insects. Most are oxidase negative, catalase positive and reduce nitrate to nitrite. Acid and gas are produced during fermentation of D-glucose, other carbohydrates and polyhydroxyl alcohols (Brenner and Farmer, 2005).

Newton *et al*. (1977) isolated *Enterobacter liquefaciens*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Citrobacter* spp. and *Serratia* spp. from 85 hides. Oppong *et al*. (2006) isolated *Citrobacter freundii* and *Proteus vulgaris* from both raw and soaked hides. In the study of Shede *et al*. (2008), *Escherichia coli, Proteus mirabilis*, *Shigella boydii*, *Photorhabdus luminescens, Pantoea agglomerans* were isolated from raw buffalo hides.

Detection of *Ewingella* in vacuum-packaged meat, vegetables, molluscs, shiitake, button and oyster mushrooms, and some clinical specimens taken from blood, wounds, respiratory tracts was reported by the investigators (Muller et al.,1995; Hamilton-Miller and Shah, 2001; Reyes *et al*., 2004). *Ewingella americana* uncommonly causes infection in humans, is associated with nosocomial infections (Grimont *et al*., 1983; Farmer *et al*., 1985; Heizmann and Michel, 1991). *Kluyvera intermedia,* which is a potential pathogen, was isolated from potable water, surface water, soil, human samples such as stool, bile and gall blader (Pavan *et al*., 2005). This organism may cause blood infections, urinary tract and soft tissue infections and septic shock (Janda, 2006).

Antimicrobial agents are commonly used for human and animal health and welfare. Antimicrobial resistance influenced by widespread and inappropriate usage of antimicrobial agents in medicine, veterinary medicine and agriculture is a global public

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and animal health concern (Madigan *et al*., 2015; World Organization for Animal Health, 2015). Antibiotic resistance profiles of some members of Enterobacteriaceae isolated from salted hide and skin samples were examined in our previous study. In that study 70% of the hide and 68% of the sheep skin strains were found to be resistant to three or more of 24 antimicrobial agents. Resistance of the isolates to ampicillin (45%), cefoxitin (20%), kanamycin (9%), ceftriaxone (45%), meropenem (2%), aztreonam (71%), ceftazidime (33%), imipenem (4%), tetracycline (16%), amikacin (5%), ciprofloxacin (5%), cephalothin (16%), gentamicin (5%), amoxycillin-clavulanate (25%), tobramycin (13%), ampicillin-sulbactam (29%), piperacillin-tazobactam (38%), ofloxacin (2%), cefuroxime sodium (45%), chloramphenicol (35%),streptomycin(9%), sulfamethoxazole/trimethoprim (25%) and nalidixic-acid(42%) was detected (Ulusoy, 2014; Birbir *et al*., 2016).

Although there are several studies on members of the family *Enterobacteriaceae,* antibiotic susceptibilities of *Ewingella americana* and *Kluyvera intermedia* isolated from soaked cattle hides and sheep skin samples treated with antimicrobial agent have not been examined yet. Hence, the goal of the study was to present phenotypic characteristics and antibiotic susceptibilities of *Ewingella americana* and *Kluyvera intermedia.*

MATERIAL AND METHOD

Test Isolates

The isolates were obtained from the soaked cattle hides and sheep skin samples using Eosin Methylene Blue Agar. Phenotypic characterization of these isolates were performed according to catalase and oxidase activities, reduction of nitrate to nitrite and API 20E test kit containing biochemical tests such as β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, utilization of citrate, production of H_2S, urease, tryptophan deaminase, production of indole from tryptophan, Voges-Proskauer, production of gelatinase, fermentation of glucose, mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose (Yazici and Birbir, 2018).

Antibiotic Susceptibility Test

One isolate of *Ewingella americana* and two isolates of *Kluyvera intermedia* were grown on Mueller Hinton Agar at 37°C for 24 hours and the isolates were separately inoculated into Mueller Hinton Broth and incubated at 37°C for 24 hours. Then, the bacterial density of these isolates was adjusted to approximately $1x10^8$ cfu/mL. Disc diffusion susceptibility method was used to detect antibiotic susceptibilities of the isolates (CLSI, 2014; EUCAST, 2014). 24 antimicrobial agents belonging to 9 categories such as tetracyclines (tetracyline), carbapenems (imipenem, meropenem), aminoglycosides (amikacin, kanamycin, tobramycin, streptomycin, gentamicin), monobactams (aztreonam), penicillins (β-lactam/β-lactamase inhibitor combinations) (piperacillin/tazobactam, amoxicillin/clavulanate, ampicillin/sulbactam, ampicillin), cephalosporins I, II and III (cefoxitin, cefuroxime sodium, ceftazidime, cephalothin, ceftriaxone), quinolones and fluoroquinolones (norfloxacin, ofloxacin, nalidixic acid, ciprofloxacin), amphenicols (chloramphenicol) and sulfonamides, dihydrofolate reductase inhibitors combinations (sulfamethoxazole/trimethoprim), were utilized in the present study. The antimicrobial agents were placed on the Mueller Hinton Agar plate

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inoculated with test isolate. After incubation at 37°C for 24 hours, the zones of growth inhibition around each of the test antibiotics were measured and the results were evaluated according to the criteria explained by the of Clinical and Laboratory Standards Institute (CLSI, 2014) and European Committee on Antimicrobial Susceptibiliy Testing (EUCAST, 2014). The test antimicrobial agents were purchased from Oxoid (Basingstoke, Hants, UK).

RESULTS AND DISCUSSION

While *Ewingella americana* was isolated from only soaked hide (CH10), *Kluyvera intermedia* was isolated from both soaked hide and skin samples (SS3 and CH1). Although *Ewingella americana* showed positive reactions of β-galactosidase, Voges-Proskauer, citrate utilization, catalase, nitrate reduction to nitrite, acid production from glucose and mannitol, negative reaction was detected in H_2S production, oxidase, urease, gelatinase, tryptophan deaminase, indol production, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase, acid production from inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose (Yazici and Birbir, 2018). These biochemical test results were similar to the results mentioned in Janda (2006).

Ewingella americana was resistant to aztreonam, ceftazidime and ceftriaxone. While *Ewingella americana* was intermediate susceptible to cefuroxime sodium, ampicillin, nalidixic acid, tetracyline and chloramphenicol, this isolate was susceptible to tobramycin, gentamicin, kanamycin, streptomycin, amikacin, meropenem, imipenem, cephalothin, cefoxitin, ampicillin/sulbactam, amoxicillin/clavulanate, ciprofloxacin, piperacillin-tazobactam, sulfamethoxazole-trimethoprim, norfloxacin and ofloxacin in the present study. Bukhari and colleagues (2008) mentioned that *E. americana*, which was isolated from a patient, was resistant to ceftazidime and ceftriaxone. Our antibiotic results were similar to that study (Bukhari *et al*., 2008). Resistance of this isolate to ceftriaxone and aztreonam was stated by Pound *et al*. (2007). Moreover, Pound *et al*. (2007) detected that *E. americana*, isolated from a patient with a chronic pulmonary disease, was also resistant to amikacin, gentamicin, tobramycin, ampicillin, ampicillin/sulbactam, cefoxitin, ciprofloxacin, imipenem, and tetracycline. While the isolate was found to be intermediate susceptible to piperacillin/tazobactam, ceftazidime, susceptible to sulfamethoxazole/trimethoprim (Pound *et al*., 2007). In our study, the susceptibility of *E. americana* against aminoglycosides, carbapenems, fluoroquinolones, sulfamethoxazole/trimethoprim and cefoxitin were consistent with antibiotic test results of Stock *et al*. (2013). The researchers emphasized that *E. americana* has natural susceptibility to most antibiotics including aminoglycosides, aztreonam, cefepime, cefotaxime, trimethoprim/sulfamethoxazole, carbapenems, cefoxitin, fluoroquinolones, tetracyclines and chloramphenicol. In another study, all hide isolates of *Ewingella americana* were susceptible to tobramycin, gentamicin, kanamycin, streptomycin, amikacin, cefoxitin, imipenem, meropenem, piperacillin-tazobactam, ofloxacin, amoxicillin/clavulanate, norfloxacin, ciprofloxacin, cephalothin, ampicillin/sulbactam and sulfamethoxazole-trimethoprim, these isolates were detected as resistant to aztreonam, ceftazidime and ceftriaxone. *Ewingella americana* was found to be intermediate susceptible to tetracyline, nalidixic acid, ampicillin, cefuroxime sodium and chloramphenicol (Birbir *et al*., 2016). Although *Kluyvera intermedia* exhibited positive reactions of β-galactosidase, ornithine decarboxylase, catalase, nitrate reduction To nitrite, acid production from glucose, arabinose, amygdalin, mannitol, sorbitol and melibiose, negative reaction was detected in citrate utilization, H_2S production, oxidase,

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urease, tryptophan deaminase, gelatinase, arginine dihydrolase, lysine decarboxylase, Voges-Proskauer, indol production, acid production from inositol and sucrose (Yazici and Birbir, 2018).

In this study, *Kluyvera intermedia* was resistant against streptomycin, cephalothin, aztreonam, ampicillin but susceptible against piperacillin-tazobactam, tobramycin, gentamicin, meropenem, cefoxitin, nalidixic acid, ampicillin/sulbactam, tetracyline, norfloxacin, ofloxacin, cefuroxime sodium, sulfamethoxazole-trimethoprim. This isolate was found to be intermediate susceptible to amikacin, kanamycin, chloramphenicol, amoxicillin/clavulanate, ceftriaxone, ciprofloxacin, imipenem and ceftazidime.

In another study, resistance of three *Kluyvera intermedia* strains, isolated from sink and distinct taps in an intensive care unit in Brazil, against meropenem, imipenem, ertapenem, doripenem was stated by Ribeiro *et al*. (2014). It was stated that two *Kluyvera intermedia* strains were susceptible against ceftriaxone, cefepime and aztreonam. In our study, resistance of *K. intermedia* against aztreonam, susceptibility to meropenem and intermediate susceptibility to imipenem and ceftriaxone were detected.

CONCLUSIONS

This is the first report that investigates antibiotic susceptibility profiles of *Ewingella americana* and *Kluyvera intermedia,* isolated from soaked cattle hides and sheep skins, against 24 different antimicrobial agents in leather industry. While *E. americana* was resistant against three antibiotics (aztreonam, ceftazidime and ceftriaxone), *K. intermedia* was resistant to four antibiotics (streptomycin, cephalothin, aztreonam, ampicillin). Due to resistance of these isolates against the antibiotics used in both human and veterinary medicine, we suggest effective antibacterial applications in leather industry to eradicate these antibiotic resistant microorganisms.

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