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Prevalence and molecular characterization of *β-lactamase* resistance gene in multidrug resistance bacteria, *Proteus spp.*

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Abstract

Existing of drug resistance bacteria in meat is a series health concern and b-lactamase is responsible to generate multi drug resistances in bacteria. Meat is a source of delivering food born pathogen bacteria including Proteus species. Recently Proteus bacteria developed drug resistance against many antimicrobial drugs and it causes difficulty in patient's treatment. Hence its important to indicate the rate of Proteus species, P. mirabilis and P. Vulgaris, in meat of different animals and to find the prevalence of b-lactamase resistance genes (blaTEM-1, blaCMY, blaCMY2, blaShv, blaOXA, and blaCTX) in Proteus species. Molecular identification of Proteus bacteria was confirmed by PCR amplification of part of 16S rRNA using Proteus specific set of primers. 70 meat samples (cattle, sheep, chicken, turkey, goat, and fish) were collected in local meat shops in the center of Sulaimani city. 29 (41.4%) samples were positive to Proteus species and 22 (75.87%) isolates were P. mirabilis and seven (24.13%) were P. vulgaris based on conventional biochemical tests. The drug sensitivity test was performed for all isolates using a disk diffusion assay (Kirby Bauer test). The multi drug resistance were found in all isolates and the most common drug resistance phenotype were against tetracycline, rifampin, and doxycycline, while the imepenem, tobramycin, and meropenem remain more affective against the bacteria. Resistance genes, blaTEM-1 and blaShv were found in five

isolates (17.2%) of Proteus. Three isolates (10.3%) were positive to blaTEM-1 resistance gene and two isolates (6.8%) were positive to blaShv. All resistance genes recorded in this study were recovered in P. mirabilis and none of them was reported in p. vulgaris. None of the isolates was positive to b-lactamase genes, blaCMY, blaCMY2, blaOXA, and blaCTX.

Keywords: Meat born pathogen, *Proteus* spp., Antibiotic sensitivity test, Resistance genes, *β-lactamase.*

1. INTRODUCTION

Increasing bacterial resistance against common antibacterial drugs is a series health concern and appearing and exchanging antimicrobial resistance among different bacteria makes a complex problem. Bacteria gain resistance because of different factors and the most common one is existing of drug resistance genes and improper using of antibiotics [1]. In animal industry, antibiotic has been used to promote animal growth in addition to therapeutic and prophylactic purposes [2]. The development of drug resistance in pathogen bacteria that due to the heavy consuming of antibacterial medicines is very well studied. Animal meat is a good source of nutrition to bacteria, so it becomes a grate reservoir of pathogenic bacteria including *Salmonella* species, *Shiga* toxin producing *E. coli* strains, and *Proteus* [3, 4, 5].

The zoonotic properties of food born bacteria in combination with producing toxin and bearing resistance genes accelerate the seriousness of the diseases and even death in untreatable cases. Millions of cases that resulted from food born pathogens have been recorded yearly globally in sporadic patients and even in outbreak cases in some countries. The outcome of the diseases can be seen in appearing of a million diarrhea symptom in children due to enteropathogenic bacteria and about three million death casualties annually [5].

Beta-lactam antibiotics are a commonly used antibiotics against different gram negative bacteria. AmpC β -lactamases is an enzyme generated by bacteria and it woks to lyse beta lactam class antibiotics. AmpC betalactamse has been studied well for more than forty years since a drug resistance gene discovered in gram negative bacteria against beta-lactam. Bacteria produce betalactamse to fight against beta lactam antibiotic as a strategy to survive [6, 7]. Its important to study and investigate AmpC beta lactamase, specifically in medical aspects because of its ability to deactivate many types of commonly used antibacterial including cephalosporins, penicillins, cephamycins, oxyimino-cephalosporins, and monobactams [6].

The disability of extended spectrum beta lactamases (ESBL) inhibitor clavulanic acid against betalactamse gene favour bacteria to have stronger resistance pattern against ESLB and beta lactam antibacterial drug. AmpC β –lactamase proteins can be expressed from either chromosome or plasmid. Many bacteria express the enzyme chromosomally including *Shigella*, *Enterobacter*, *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter freundii*, *Hafnia alvei*, *Pseudomonas aeruginosa*, *Morganella morganii*. *Escherichia coli*, and *Acinetobacter species* Laghawe Avinash et al., 2012, [7, 8, 9].

The plasmid bearing AmpC beta lactamse gene is expressed constitutively, while some of them expressed through beta lactam induction similar to chromosomal gene. The spreading of plasmid bearing AmpC resistance gene is easier than chromosomal gene and this makes plasmid AmpC beta lactamase gene to be clinically more important. Enterobacteriacae exchange plasmid bearing resistance gene continuously to develop resistance against beta lactam antibacterial medicines such as in *Salmonella* spp., *Klebsiella* spp., *Proteus mirabilis*, and *Escherichia coli* [10].

In some gram negative bacteria, the occurrence of AmpC beta lactamase is high but its not well studied in *Proteus* species especially in sulaimani city. *Proteus* recently shows resistance against many antibacterial medicines and this cannot be detected accurately through regular antibiotic sensitivity test phenotypically and its not guided by Clinical and Laboratory Standards

Institute (CLSI) until now [11, 12].
Polymerase chain reaction (PCR) has been developed to amplify and rapid identification of resistance genes [13]. This method can be used to detect six different beta lactamase genes in gram negative bacteria, *Proteus* species including *bla*TEM-1, *bla*CMY, *blaCMY2*, *bla*Shv, *bla*Oxa, and *bla*CTX [13, 14, 15]. The aim of this study was to 1- finding the rate of *Proteus* species in raw meat of different animals in local meat shops. 2- to find multi drug resistance pattern of *Proteus* against commonly used antimicrobials. 3- to detect the existing beta lactamase resistance genes in *Proteus* species.

2. METHODS AND MATERIALS

Sample collection
A total of 70 meat samples (Beef, Sheep, Turkey, Chicken, Fish) were collected from local meat shops in the centre of Sulaimany city from December 2018 to January 2019. Every single sample was purchased from different shop to collect a variety of samples and represent different types and sources of bacterial contamination. The samples were put into a sterile container and kept cold at 4-10°C until they are arrived to the laboratory for further processing.

Sample processing
For every sample, 10 gm of the meat sample was weighed and homogenised in 90 ml of nutrient broth using sterile blender. The homogenised meat was put into a flask container and was then incubated for 16 hours at 37 °C in shaking incubator.

Isolation and Identification of Proteus species
All culture media and biochemical tests were purchased from Accumedia LAB (Neogene Culture Media, Heywood, UK). For isolation of gram negative bacteria, *Proteus* species, approximately 20 µl of the overnight culture was taken and streaked on MacConkey`s agar and incubated at 37°C overnight.
The colonies were taken and streaked again on nutrient agar to detect swarming properties after overnight incubation at 37 °C. The samples from swarming were taken to obtain a typical colony on MacConkey`s agar. After incubation, a colonies were taken and transfer to the MacConkey`s agar containing swarming inhibition substance to further confirmation of the *Proteus* species swarming characteristic.

Molecular Identification of Proteus species

DNA Extraction
A fresh single colony was dissolved in 100 ul of sterilized distilled water and the mixture was boiled at 99°C for 15 minutes using thermo-cycler heating blocks (Applied biosystem 2727, California, USA). Two ul of the boiled bacteria was used as DNA template for PCR amplification of the gene of interest.

Polymerase Chain Reaction for the Detection of Proteus species
For specific molecular detection of *Proteus* bacteria, *16s rRNA* gene (201 bp) was PCR amplified using oligoes and protocols according to [16]. The PCR volume of 20 µl was used for PCR reaction and it was containing a mixture of 10 µl of 2X premix *RedTaq* DNA polymerase (SBSbio, Beijing, China), 0.5 µM of each primers and 2 µl of DNA template (boiled colonies). The PCR running condition was started at temperature of 94°C for 5 min, and 35 cycles of 94°C 30 Sec, 55°C 30 Sec, 72°C 30 Sec, with the final extension temperature 72°C for 7 minutes.
The PCR products were then fractionated on 1% agarose gel using DNA gel electrophoresis and visualized by blue light excitation of fluorescence dye, GoodView (SBSbio, Beijing, China) using SmartDoc 2.0 Imaging System (Accuris, NJ, USA)

Identification of Proteus species, P. vulgaris and P. mirabilis

After PCR confirmation of *Proteus* bacteria, Simmons citrate test was used to identify *Proteus* species. A fresh single colony was incubated into a slant agar of Simmons citrate and the culture was incubated aerobically at 37°C for 24 hours. The selection depended on changing color from green to blue for positive biochemical reaction (citrate utilization) in the presence of *Proteus mirabilis* and not color change (no citrate utilization) for negative result in the case of *P. vulgaris* [17].

Antibiotic sensitivity test

Antibiotic sensitivity test was performed using Kirby Bauer test (disk diffusion test) according to the protocol described by the CLSI (2002) [12]. Antibiotic sensitivity test was performed for 13 different antimicrobials as follow, amoxicillin (AX 25), Tobramycin (TOB 10), amoxicillin-clavulanic acid (AMC 30), Tetracycline (TE 10), Doxycycline (DO 10), Imipemem (IMP 10), trimethoprim/sulfamethoxazole (SXT 25), meropenem (MEM 10), Ciprofloxacine (CIP 10), gentamycin (CN 10), Rifampin (RA 5), amikacin (AK 10), and cefotaxime (CTX 30).

The antibiotic disks were purchased from Bioanalyse Pharmaceutical (Bioanalyse, Ankara, Turkey) and the results were interpreted according to the clear zone diameter measured around the disc according to the manufacturer chart.

Polymerase Chain Reaction of β-lactamase resistance genes

PCR amplification for six *β-lactamase* resistance genes (*bla*TEM-1, *bla*CMY, *blaCMY2*, *bla*Shv, *bla*Oxa, and *bla*CTX) was carried out using multiplex PCR. Multiplex PCR was divided into two groups, G1 were *bla*TEM-1, *bla*CMY, *blaCMY2*, and G2 were *bla*Shv, *bla*Oxa, and *bla*CTX. The universal primers were taken and used according to [15, 18]. The PCR mixture was carried out in 20 µl volume reaction containing 10 ul 2X *RedTag* DNA polymerase premix, 0.2 µM of each primer, and 2 µl of DNA extracted from boiled colonies. The PCR was run under the following condition, it started with 94°C for 5 min, and 35 cycles of 94°C 30 Sec, 55°C 30 Sec, 72°C 30 Sec, with the final extension temperature 72°C for 7 minutes using thermo cycler (Applied Biosystems 2727, Calif., U.S.A.).

The reaction DNA product was resolved on 1% DNA agarose gel, stained with GoodViw fluorescence DNA stain (SbsBio, Beijing, china) and visualized under blue light transilluminator using SmartDoc 2.0 Imaging System (Accuris, NJ, USA).

3. RESULTS

Prevalence of Proteus vulgaris and Proteus mirabilis

A total number of 70 meat samples were processed. The samples were collected from different animal and organs including beef 10 samples, sheep 10 samples, goat 10 samples, turkey 10 samples, fishes 10 samples, chicken 10 samples, and chicken liver 10 samples. Out of these samples 29 (41.4%) isolates were confirmed as *Proteus* bacteria by swarming properties of *Proteus* and specific PCR confirmation of *16S rRNA* (201 bp) (Figure 1). Meat is a good source of nitrogen, carbon and energy so it considers as a good reservoir for pathogen bacteria. Many pathogen bacteria can grow on meat and become a source of food born bacterial diseases in human.

Simmons citrate test was undertaken to identify two species of *Proteus*, *P mirabilis* and *P. vulgaris*, based on ability of bacterial species to utilize citrate. *P. mirabilis* recorded the highest rate in meat (22 isolates, 75.87%) in compare to *P. vulgaris* (seven isolates, 24.134%) (Figure 1).

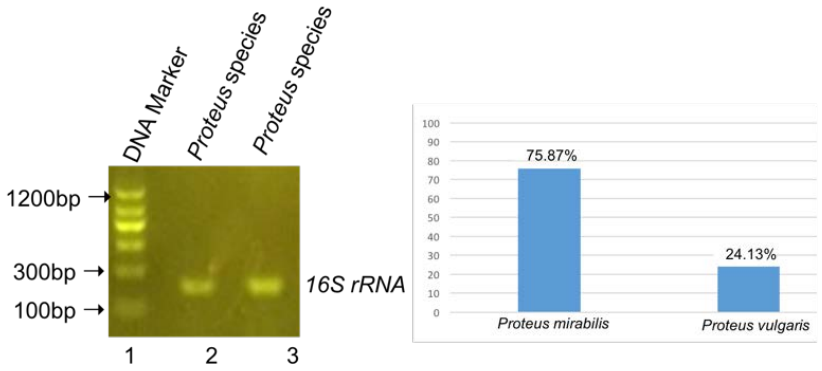

Figure 1. Identification and the rate of *Proteus* species in raw meat. PCR confirmation of Proteus bacteria (The left image). The percentage of *P. mirabilis* and *p. vulgaris* in raw meat (The right image).

Antibiotic Susceptibility Examination

Antibiotic susceptibility test was carried out using Kirby-Bauer disc diffusion test on Muller Hinton agar. The pattern of Antibiotic Susceptibility testing of isolates were different in their susceptibility against 13 common antibacterial discs (Figure 2). All isolates showed 100% resistance to three antimicrobials, tetracycline, rifampin, and doxycycline. Multi drug resistance were found in all isolates for many types of antimicrobial especially tetracycline, rifampin, and doxycycline, and tobramycin. The overall antibiotic resistance pattern of *Proteus* bacteria was like the following; tetracycline (100%), rifampin (100%), and doxycycline (100%), gentamycin (65.5%), tobramycin (62%), amoxicillin (93.1%), amoxicillin-clavulanic acid (82.7%), Imepemem (68.9%), trimethoprim/sulfamethoxazole (93.1), meropenem (65.5%), Ciprofloxacine (68.9%), amikacin (65.5%). Amikacin, imepenem, and ciprofloxacin remained active against *Proteus* and most of *Proteus* species showed the highest rate of susceptibility to them.

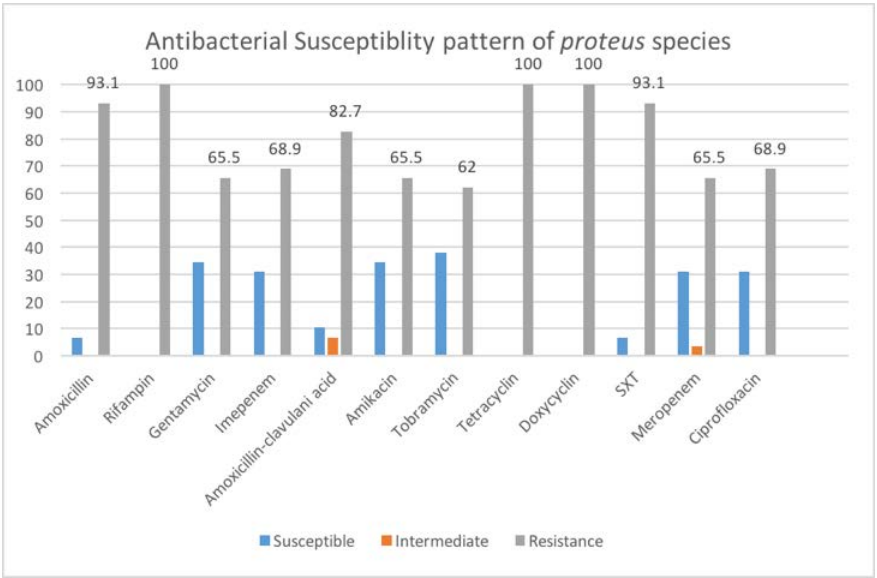

Figure 2. Antibiotic susceptibility pattern of Proteus species isolated from different raw meat in local meat shops in Sulamiani city.

Prevalence of β-lactamase resistance genes

Existing of ***β-lactamase*** genes in gram negative bacteria enable the bacteria to express β-lactamase enzyme to hydrolyze and deactivate common β-lactam ring of antibiotics, cephalocporins and ampicillin [6]. This leads to developing bacterial resistance against these antibiotics. Among ***β-lactamase*** resistance genes, SHV, CTX-M, TEM, CMY, CMY2, and OXA are the most common and well documented [19]. This study investigated the existence of these resistance genes in *P. mirabilis* and *P. vulgaris* by PCR amplification of their conservative region using gene specific set of primers.

blaCMY, blaCMY2, blaOxa, and blaCTX

PCR amplification was carried out to find different *β-lactamase* resistance genes using universal gene specific primers. All *Proteus* isolates showed negative result to four types of *β-lactamase* resistance genes, *bla*CMY, *blaCMY2*, *bla*Oxa, and *bla*CTX. So none of the isolates carry these types of resistance genes on their chromosome and plasmids.

***bla*TEM**

Out of 29 *Proteus* isolates, three isolates (10.3%) were positive to one type of *β-lactamase* resistance genes, *bla*TEM (1080 bp) (Figure 3). All three resistance genes were found in *P. mirablis* (100%) and *bla*TEM was not recorded in *P. vulgaris* (0%). Two isolates bearing *bla*TEM showed multidrug resistance to many beta lactam antibiotics, amoxicillin and amoxicillin-clavulanic acid and one isolates showed the resistance pattern to another new generation of β-lactam of antibiotics, cefotaxime.

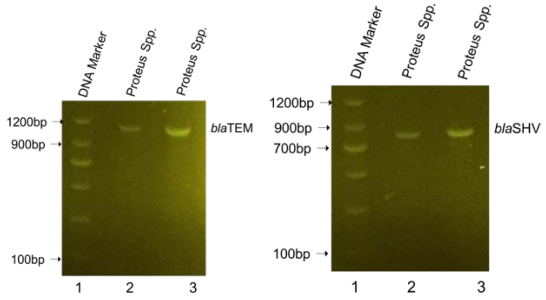

Figure 3. PCR amplification of blaTEM and blaSHV resistance genes in Proteus species. The amplicons were resolved on 1% DNA agarose gel and the size of the genes were compared to the DNA ruler of 1.2 Kb.

***bla*SHV**

*bla*SHV resistance gene (797 bp) was found in two *Proteus* isolates (6.8%) (Figure 3). All positive isolates to *bla*SHV belonged to *P. mirabilis* (100%). One of the *P. mirabilis* expressing the enzyme of *bla*SHV gene became resistance against amoxicillin-clavulanic and cefotaxime, and amoxicillin, but the second isolate is still sensitive to extended spectrum and new generation of β-lactam, amoxicillin-clavulanic acid and cefotaxime, respectively.

4. DISCUSSION

Meat of animal is sterile and does not contain any bacteria [20], but it can be contaminated after slaughtering. The sources of meat contamination are variable starting from post slaughter processing until it arrives to the markets and home kitchens. Bacterial contamination may come from intestinal content of the the animal during visceral removal or sources from environment including butcher's hands, tools and transportation equipments [21, 22, 23]. Meat is a good source of food, carbon, nitrogen and energy, for bacteria growth and meat bearing pathogens leads to transmitting of pathogens to human, causeing infection and intoxication [24]. *Proteus* is one of the bacterial pathogens that transmit from animal meats and causes infection in human.

It has been found through different studies that food born *Proteus* leads to diseases in human such as UTI, rheumatic arthritis, and hospital acquired wound infection [25, 26, 27].

In this study, the rate of Proteus in animal meat was 75.8% and it shows the risk of zoonotic diseases in Sulaimani city. Meat of different animals contains Proteus species and it shows the existence of zoonotic pathogen in markets and makes a public health concern. The common type of Proteus in meat in Sulamani city was *P. mirabilis*, 75.8%. *Proteus mirabilis* is one of the common causes of a hospital acquired human infection among other *Proteus* species so it attracts a public health concern more than other species [27]. This study agrees with other international studies that *P. mirabilis* is the most common species in raw meat of different animals [28, 29, 30, 31].

Isolates of Proteus in this study showed a complete resistance (100%) to the most common antibiotics, tetracycline, rifampin, and doxycycline. In addition, multi drug resistance were found in all isolates for many types of antimicrobial especially tetracycline, rifampin, and doxycycline, and tobramycin. A high percentage of bacterial resistance towards tetracycline is comparable with previous studies [32, 33]. Appearing drug resistances against trimethoprim/sulfamethoxazole) and ciprofloxacine is of significantly important because they are known to be used as common and very affective antibacterial agents against gram negative bacteria [19]. Similar multi drug resistance have been found against other gram negative bacteria globally [34, 35]. Moreover, resistance was found in this study against common β-lactam antibiotics, amoxicillin, amoxicillin-clavulanic acid. This is significantly important because these antibiotics are used against wide ranges of Proteus infection in human [36].

The existence of β-lactamase gene is significantly important and it's a health concern. This enzyme destroys β-lactam ring of antibiotics and enable the bacteria to resist common β-lactam ring antibiotics. Six of these resistance genes were known as common β-lactamase resistance genes, (*bla*TEM-1, *bla*CMY, *blaCMY2*, *bla*Shv, *bla*OXA, and *bla*CTX) [19] and they were investigated in this study in Proteus species. A specific set of primers used to amplify these genes specifically using polymerase chain reaction in two groups of multiplex PCR. Out of 29 Proteus isolates, three isolates (10.3%) were positive to *bla*TEM and two isolates (6.8%) were positive to *bla*SHV resistance genes. All five resistance genes were found in *P. mirablis* (100%) and none of them was recorded in *P. vulgaris* (0%). None of the other four resistance genes, *bla*CMY, *blaCMY2*, *bla*Oxa, and *bla*CTX, was reported in this study. Isolates bearing *bla*TEM and *bla*SHV showed multidrug resistance to many beta lactam antibiotics, amoxicillin and amoxicillin-clavulanic acid cefotaxime. So in this study, *bla*TEM and to *bla*SHV were the commonly reported resistance genes are in Proteus species and they exist only in *P. mirabilis.*

The rate of β-lactamase genes is less common in *Proteus* species than in other gram negative bacteria [37, 38]. This study agrees with previous finding to report the presence of β-lactamase genes in Proteus species [39, 40]. In general, there were no β-lactamase resistance genes reported in many studies [37] or its less commonly reported [34], but five isolates of Proteus (17.2%) were found in Sulaimany city. This is clinically importance and it is alarming because it hits the value of many β-lactam antibiotics and impose the patients to face difficulty in treatment. This study strongly agrees with the finding in Karbala city in Iraq which reported the highly occurrence of both resistance genes, *bla*TEM and *bla*SHV, in *Proteus mirablis* [41].

5. CONCLUSION

The result of the current study showed that the rate of contamination of meat with *Proteus* species was 41.1% and the most common species is *Proteus mirabilis*. *Proteus* showed a complete resistance to many antibacterial agents and multi drug resistanc including tetracycline, rifampin, and doxycycline. Bacteria showed less resistance to imepenem, tobramycin, gentamycin, and meropenem. Both β-lactam resistance genes, *bla*TEM and to *bla*SHV, were found in Proteus species and *bla*TEM is the most common one. All β-lactam resistance genes found in this study was reported only in *P. mirabilis*. None of the Proteus isolates were positive to *bla*CMY, *blaCMY2*, *bla*OXA, and *bla*CTX.

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