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Comparative antibiotic resistance profile of *Acinetobacter spp.* isolated from fish, chicken and beef meat

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Abstract

Acinetobacter is a gram-negative bacterium which is found to be involved in serious health implications due to consumption of contaminated food. In present study different markets of Lahore were selected to collect fish, beef and chicken meat samples. Identification was performed by using microscopic, morphological, biochemical and molecular analysis of all the samples. Sample were subjected to antibiotic resistance to create a comparative profile for antibiotic resistance for fish, beef and chicken meat. In the present study the molecular identification was used for carbapenem resistance gene (SPM-1). The results indicated that 24 (26.6%) of total samples were positive for *Acinetobacter* species. The prevalence of the *Acinetobacter*species was (30%) (26.6%) and (23.3%) in fish meat, chicken meat and beef, respectively. Ampicillin and trimethoprim showed highest resistance 100% followed by tetracycline 100%, 75% and 66.6% in beef, chicken and fish isolates respectively. Only 17 of 24 isolates were confirmed as targeted bacterium for the SPM-1 gene by PCR. *Acinetobacter* species had low percentage in beef meat than in fish meat and chicken meat. The chicken meat is contaminated with antibiotics resistance *Acinetobacter species*, they can cause serious diseases in future in poultry, animals and human. The risk concern with antibiotics resistance *Acinetobacter* species cannot be ignored because, they resist to currently using potential antibiotics.

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Introduction

Acinetobacter are strictly aerobic, gram negative, non-fermentative rods. Their colonies are generally non-pigmented, pale yellow or grayish-white (Doughari *et al*., 2011). Found in areas contaminated with vegetables, sewage, dump places and hydrocarbons (Berlau*et al*., 1999). Soil, marine fish, freshwater fish and water (Čož-Rakovac *et al*., 2002) foods producing animals (Wang *et al*., 2012) and homo sapiens (Doughari *et al*., 2011). Different species of *Acinetobacter* had been isolated from the dairy products e.g milk, cheese, and as well as from fish and different meat samples. From Lebanon, Rafeietal. described non-*Acinetobacter baumannii* isolation including *Acinetobacter pittii*, *Acinetobacter calcoaceticus*, *Acinetobacter bereziniae,* and *Acinetobacter soli* from cow raw meat, raw milk ofcow milk, raw cheese, and from vegetable, and recently carbapenem resistant strains of *Acinetobacter species* isolated from vegetables (Rafei *et al*., 2015).The *Acinetobacter species* are related with bacteremia, meningitis, pulmonary infections, diarrhea and notorious nosocomial infections with death rates 20 to 60%. The person-to-person transmission is via contact, food contamination and water, and unhygienic hospital apparatus (Doughari *et al*., 2011). Because of availability micronutrients, minerals, vitamins, proteins for mental and physical health meat is most important source (Jalil *et al*., 2013). Across the world poultry meat and fish meat is of great concern because of their storage in freeze conditions (0–10°C) only (Chouliara *et al*., 2008). Psychrophiles of different genera such as *Acinetobacter*, *Lactobacillus*, *Moraxella*, *Pseudomonas*, *Microbacteria*, *Klebsiella, Vibrio and* Brochotrix are more related to poultry and fish meat (Gill and Newton, 1978). *Acinetobacter* strains from three decades have developed resistance against newly developed antibacterial agents and MDR *Acinetobacter baumannii* is identified. They are leading cause of hospital acquired infections and are prevalent across the world (Abbo 2005). Antimicrobial genes are transmitted by them and are considered to have impact in aquaculture (Kozińska *et al*., 2014). They are referred to exhibit resistance against atleast three different classed antimicrobial agents. e.g. all groups of cephalosporins, penicillins and to fluoroquinolones and aminoglycosides (Jung and Park, 2015). More than two decades ago it was reported that transposons, plasmids and integrons are main components for transmission of resistance (Fournier *et al*., 2006). The antimicrobial resistances of sulphonamides, tetracycline, erythromycin trimethoprim and ciprofloxacin have been testified among *Acinetobacter species* from marine environments (Guardabassi *et al*., 2000). Antibiotic resistance hinders in provision of actual treatment but also upturns morbidity and mortality (McDonald, 2006).Misuse and over use of antibiotics in poultry and fisheries lead to antibiotic resistance by producing residue in muscles, and also produce many complications. In several low-income countries the surge of antibiotic resistance has been observed and are very disastrous because of expensiveness of antibiotics available (Eliopoulos *et al*., 2003).

In Pakistan, *Acinetobacter* spp resistance is a serious health issue. Contaminated food consumption is among serious issues resulting in serious clinical implications. *Acinetobacter* are resistant themselves as well as are involved in spread of genes responsible for resistance to sulphonamides, erythromycin, trimethoprim, tetracycline and ciprofloxacin.The aim of the current research work was to find out the comparative antibiotic resistance profile of *Acinetobacter spp* isolated from fish, chicken and beef meat.

Material and methods

Sample collection

A total of 90 meat samples were collected randomly from local retail meat shops and super markets of fish meat, chicken meat and beef meat. The test samples were taken in sterile UV irradiated zip lock bags. The samples were transported aseptically to University Diagnostic Laboratory (UDL), UVAS, Lahore maintaining the cold chain at 4°C.

Sample processing and enrichment

The collected samples were cut and chopped with

sterile scalpel in biosafety cabinet. Sterilized distilled water was used for samples washing and sterilized surface with 3 % bleach (Antwi-Agyei and Maalekuu 2014). A full loop culture from the enrichment broth were then plated on leeds Acinetobacter medium (LAM) petri plates aseptically in biosafety cabinet and incubated at 37°C for 24 hours. Sub culturing was performed to obtain pure colonies and then sub cultured on MacConkey agar and Blood agar plates. The samples were subjected to standard microbiological identification tests based on colony morphology and biochemical tests for confirming their identity (Cheesbrough *et al.* 2006).

Identification of *Acinetobacter species*

All collected samples were first inoculated into baumannii medium, a selective enrichment mineral medium, after 24 hour they were cultured on Leeds *Acinetobacter* medium. The plates having mauve pink color colonies were assumed to be positive.

Morphological identification of Acinetobacter species

Morphological identification of *Acinetobacter species* was done via gram staining. Under microscope. Red color gram negative coccobacilli in pairs were suspected to be *Acinetobacter species*.

Biochemical identification of Acinetobacter species

Biochemical tests were performed for identification of *Acinetobacter species*. The biochemical tests comprised oxidase, catalase, TSI, Motility, indole, methyl red, vogues prousker, citrate utilization test and urease for the identification of bacterial isolates. The Bergey's Manual of Systemic Bacteriology was used for the identification as a reference manual (Vos*et al.*, 2011).

Antimicrobial susceptibility patterns of isolated Acinetobacter species

Antibiotic susceptibility pattern of the identified isolates was investigated by disk diffusion Kirby–Bauer method, according to the Clinical and Laboratory Standards Institute (CLSI) (2016). Two to three purified colonies of *Acinetobacter spp.* were transferred into 10 ml sterilized normal saline (0.93%) and density of these suspensions was adjusted to 0.5 McFarland standards. The adjusted suspensions were spread by sterilized cotton swab on separate plates of Mueller-Hinton Agar (Oxoid).

The susceptibility of the *Acinetobacter species* isolates to different antimicrobial agents are: Ampicillin (10 µg), Tetracycline (30 µg), chloramphenicol (30 µg), Imipenem (10 µg), Gentamicin (30 µg) Norflaxacin (5 µg), Sulfa-methaxazole (30 µg) Trimethoprim (30 µg), kanamycin (10 µg), and ceftriaxone (30 µg).

Molecular Confirmation

Pure colonies of *Acinetobacter species* were confirmed by Polymerase chain reaction targeting the SPM-1 gene of carbapenamase enzyme.

Polymerase chain reaction

The DNA extraction was done by using *GF-1*vivantis kit. SPM-1 gene of *Acinetobacter species* was targeted for the molecular confirmation of carbapenems resistance in isolated organism. PCR amplification was performed by mixing the template DNA, primers, nuclease free water andeconoTaq 2X Master Mix by Lucigen Company (Wisconsin USA).

The PCR reaction conditions were set for 36 cycles. Initially denaturation was done at 94°C for 5 minutes. Then for 36 cycles, denaturation temperature was set at 94°C for 40 seconds. The temperature was then allowed to fall to 55°C for 40 seconds. At this temperature the primers bind to the target DNA. Then the temperature was again increased to 72°C for 40 seconds to – provide condition for DNA polymerase to amplify the target DNA. These three steps were repeated for 36 times and then temperature raise to 72°C to finally amplify the DNA fragments for 5 minutes.

Agarose gel electrophoresis

To confirm the presence of DNA, the amplified DNA was run on agarose gel electrophoresis using the standard protocol (Lee *et al.* 2012). The separation of DNA fragment was done by gel electrophoresis at

120V for 30 minutes and bands of the PCR products on gel were visualized by UV transilluminator.

Statistical analysis

The data was compiled in MS Excel and were analyzed by chi square test and kruskal-wallis test using SPSS software version 21.

Results and discussion

The present study was planned to determine the contamination of *Acinetobacter species* and its antibiotics resistance level in fish meat, chicken meat and beef meat collected from local and supermarket of Lahore city. Out of 90 samples 24 (26.6%) were positive and 66 (73.3%) were found negative.

The identified isolates of *Acinetobacter spp,* were subjected to antibiotic resistance profile and the phenotypic carbapenem resistance were further confirmed through gradient PCR.

Table 1. Samples distribution.

Type of meat samples	Source of meat samples		
	Local market	Super market	Total
Fish	15	15	30
Chicken	15	15	30
Beef	15	15	30
Total	45	45	90

Cultural identification of Acinetobacter spp

Leeds Acinetobacter Medium (LAM) was used for the isolation of *Acinetobacter species* from meat samples of fish, poultry and beef.

On Leeds Acinetobacter Medium (LAM) pink color colonies were suspected as *Acinetobacter species*

Phenotypic identification

The purified pink colour colonies were subjected to Gram's staining for morphological identification. The microscopic examinations of the smear by the help of Gram's staining the organisms were confirmed as gram negative short rods in diploid form.

Table2. Percentage prevalence of *Acinetobacter species* isolates in different meat types.

Type of meat samples	No of samples	Status				p-value
		Positive		Negative		
		No.	Percentage	No.	Percentage	
Fish meat	30	05	33.3%	10	66.66%	
		04	26.6%	11	73.33%	

Beef	30	04	26.6%	11	73.33%	0.337*
		03	20%	12	80%	
Chicken meat	30	04	26.6%	11	73.33%	
		04	26.6%	11	73.33%	
Total	90	24	26.66%	66	73.33%	

*p-value for meat type specific prevalence calculated by Kruskal-Wallis.

Identification of Acinetobacter species via biochemical test

Oxidase test

Oxidase positive microorganisms produced enzyme cytochrome oxidase that oxidized the phenylenediamine into a deep purple colour. The suspected *Acinetobacter species* isolates were oxidase negative.

Catalase test

Catalase positive microorganisms produced enzyme catalase which detoxifies hydrogen peroxide by breaking into water and oxygen gas results in formation of bubbles. The suspected *Acinetobacter species* isolates were catalase positive.

Triple sugar iron (TSI) test

The isolates were also grown on triple sugar iron (TSI) agar.

Changes in color of butt were considered as glucose and slope as lactose fermentation, blackening of butt as hydrogen supplied production. Cracks in the media was considered as gas production.

The suspected *Acinetobacter species* isolates were non-glucose, lactose fermenter and no H_2S gas producers, but few isolates of *Acinetobacter species* showed gas production and many of the isolates did not show gas production.

Table. 3. Percentage prevalence of *Acinetobacter species* isolates in meat samples collected from different sources.

Origin of samples	No of samples	Status				p-value
		Positive		Negative		
		No.	Percentage	No.	Percentage	
Local outlets	45	13	28.9%	32	71.1%	0.634*
Super markets	45	11	24.4%	34	75.6%	
Total	90	24	26.7%	66	73.3%	

*p-value for local outlets and supermarkets of prevalence calculated by chi square test.

Motility agar

Motility test is used to differentiate motile and non-motile bacteria. The positive test was indicated by the presence of diffuse growth away from the stab line of inoculation. While negative test was only grown on stab line of inoculation. The suspected *Acinetobacter species* isolates were motility negative.

Urease test

Urease positive organisms are capable of hydrolyzing urea to produce ammonia and carbon dioxide. Urease positive were indicated by appearance of bright pink color on the urea agar plates. The suspected *Acinetobacter species* isolates were urease negative.

Table4. Comparative antimicrobial sensitivity profiles of *Acinetobacter species* isolates derived from different meat types.

	Fish meat			Chicken meat			Beef meat		
Antibiotics	n=9 (30%)			n=8 (26.6%)			n=7 (23.3%)		
	S	I	R	S	I	R	S	I	R
Ampicillin	0%	-	100%*	0%	-	100%*	0%	-	100%*
Ceftriaxone	11.1%	66.6%	22.2%	0%	75%	25%	0%	85.7%	14%
Imipenem	-	66.6	33.3	37.5	25	37.5	42.8	28.5	28.5
Gentamicin	100%	-	0%	75%	-	25%	28.5%	42.8%	28.5%
Kanamycin	44.4%	-	55.5%	37.5%	-	62.5%	42.8%	14.2%	42.5%
Tetracycline	11.1%	22.2%	66.6%	12.5%	12.5%	75%	0%	-	100%
Chloramphenicol	11.1%	-	88.8%	25%	25%	50%	14.2%	28.5%	57.1%
Trimethoprim	0%	-	100%*	0%	-	100%*	0%	-	100%*
Sulfamethoxazole	100%	-	0%	12.5%	62.5%	25%	85.7%	14.2%	0%
Norfloxacin	100%	-	0%	37.5%	25%	37.5%	57.1%	28.5%	14.2%
p-value				1.462†					

P-value for origin-based comparison calculated by Kruskal-Wallis test.

Citrate utilization test

Citrate test were used to differentiate that organism utilize citrate as a carbon source and produce alkaline compound as end product.

The organisms change medium color from green to blue were well-thought-out to be positive for citrate test. Isolates in the present work were positive for citrate utilization.

Indole test

Indole test were performed that organism produce tryptophanase for the tryptophan lysis. By the addition of kovac›s reagent red circular ring were formed on the surface of the tubes were considered as positive. The *Acinetobacter species* isolates were negative for indole test.

Methyl red test

Methyl red positive organisms can ferment glucose which results in acidic production. By the addition of methyl red reagent, the color of entire tube changed to red were considered as positive. The *Acinetobacter species* isolates were negative for methyl red test.

Table 5.PCR confirmation of carbapenem resistance gene (SPM-1) in test samples.

Sample type	Sample number(n)	Positive sample			
		Bacterial Culturing		PCR	
		No. of isolates	Percentage(%)	No. of isolates	Percentage(%)
Fish	30	9	30	5	55.55
Broiler	30	8	26.66	7	87.5
Beef	30	7	23.33	5	71.4
Total	90	24	26.6	17	70.83
\					

Vogesproskauer test

This test was used to determine the productions of non-acidic or neutral end products. After 24 hours incubation Barret reagent was added first 10 drops of Barret reagent A (Alpha naphthol) were added followed by 5 drops of Barret reagent B (40% KOH). After addition of reagent all tubes were shake gently. After 15 minutes a positive test was represented by the appearance of red color precipitate.

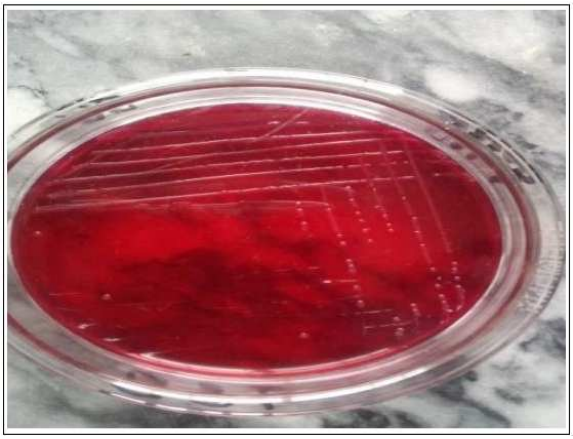

Fig. 1. Growth of *Acinetobacter* species on leedsacinetobacter medium.

Antimicrobial Susceptibility testing

The explanatory criterion was used to determine the zone diameter of inhibition of the antibiotics in millimeters (mm) against *Acinetobacter* species so that the organism was marked sensitive, intermediate or resistant. The isolate with no zone of inhibition was marked as R while the isolate having small or large zone of inhibition, its diameter was determined in millimeter.

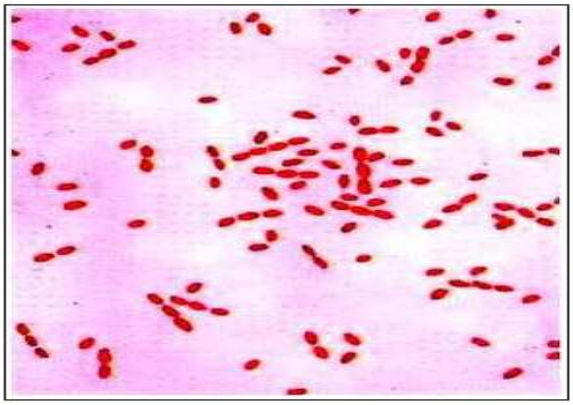

Fig. 2. Stained smear showing Gram negative short rods.

Molecular confirmation of antibiotic resistance in Acinetobacter species

Confirmation of carbapenemase resistance:Out of 24 culturally positive isolates, only 17 samples were confirmed SPM-1 gene positive by PCR.

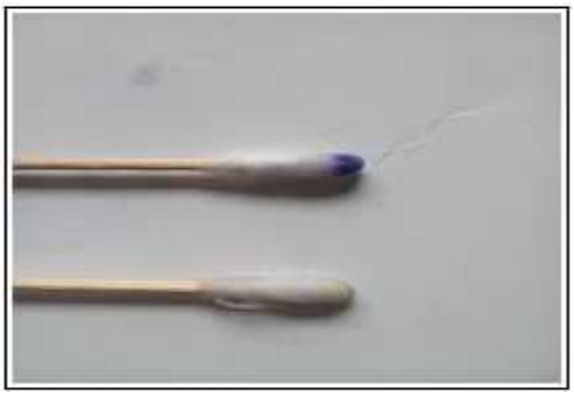

Fig. 3. Oxidase test negative.

Fig. 4. Catalase positive.

The gel picture shows carbapenem resistance gene (SPM-1) specific amplicons (size 271 bp) of fish meat, chicken meat and beef isolates. Lane M indicate 100bp DNA ladder, Lane 15 shows positive control (*Acinetobacter baumannii* ATCC) and lane 1 to 14 shows fish meat, chicken meat and beef isolates amplicons and empty lane shows PCR negative isolates of three different types of meat.

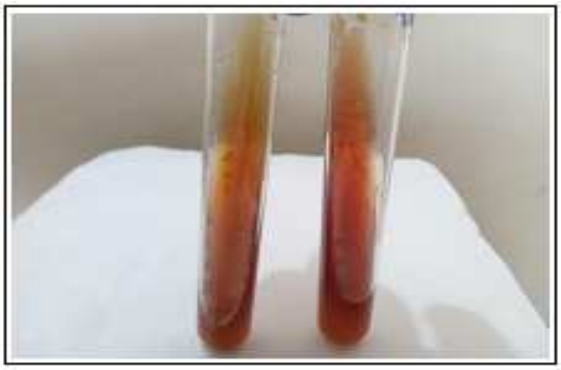

Fig. 5. TSI negative.

A study was conducted to determine the presence of *Acinetobacter species* in fish meat, chicken meat and beef meat through conventional method. Furthermore, antibiotic resistance profile of the positive isolates was also determined using different class of antibiotics which is currently used for the treatment of *Acinetobacter species*. A total of 90 samples, fish (30n), chicken (30n) and beef (30n) were collected from different local markets and super markets of Lahore.

Fig. 6. Motility tests negative.

Fig. 7. Urease test negative.

The results indicated that 24 (26.6%) of total samples were positive for *Acinetobacter species*. The prevalence of the *Acinetobacter* species was (30%) (26.6%) and (23.3%) in fish meat, chicken meat and beef respectively.

Fig. 8. Citrate utilization tests.

Moreover, the susceptibility of the isolates was tested using clinical break points of different class of antibiotics according to the Clinical and Laboratory standard institute (CLSI 2016).

Fig. 9. Indole test negative.

Fig. 10. Methyl red test for.

Ampicillin and trimethoprim showed highest resistance 100% followed by tetracycline 100%, 75% and 66.6% in beef, chicken and fish isolates respectively.

The chloramphenicol showed 88.8%, resistance in fish, 57% in beef and 50% in chicken meat. Norfloxacin showed highest sensitivity 100%, 57% and 37% in fish, beef and chicken isolates respectively. Norfloxacin followed by sulfa-methimazole showed sensitivity 100% in fish, 85.5% in beef 12.5% in chicken meat isolates. In the present study the molecular identification was used for carbapenem resistance gene (SPM-1) to confirm the resistance against carbapenem drug shows by disc diffusion method.

The results of the present study by disc diffusion method were 33.3% in response to imipenem. Our results are quite like the finding of (Moradi *et al.* 2015) as they show 32% resistance to the imipenem. The results of (Moradi *et al.* 2015) is also in favor with the investigation (Kulah *et al.* 2009) as they show (35%) resistance to the imipenem by disc diffusion method. Similarly, the positive samples were then subjected to PCR only 17 of 24 isolates were confirmed as targeted bacterium for the SPM-1 gene.

A few samples of *Acinetobacter spp,* showed resistance against imipenem by disc diffusion methods but molecularly they were negative for SPM-1 gene.

Fig. 11. Vogesproskaeur test negative.

In conclusion we can say that, *Acinetobacter species* had low percentage in beef meat than in fish meat and chicken meat. The chicken meat is contaminated with antibiotics resistance *Acinetobacter* species, they can cause serious diseases in future in poultry, animals and human.

The risk concern with antibiotics resistance *Acinetobacter species* cannot be ignored because, they resist to currently using potential antibiotics.

The present study conclusion was matching to the finding of (Phillips *et al.* 2004) they investigate that antibiotic usage in food animals, growth promotions in animals and poultry and prophylaxis use of antibiotics in aquaculture is responsible for generation antibiotics resistance.

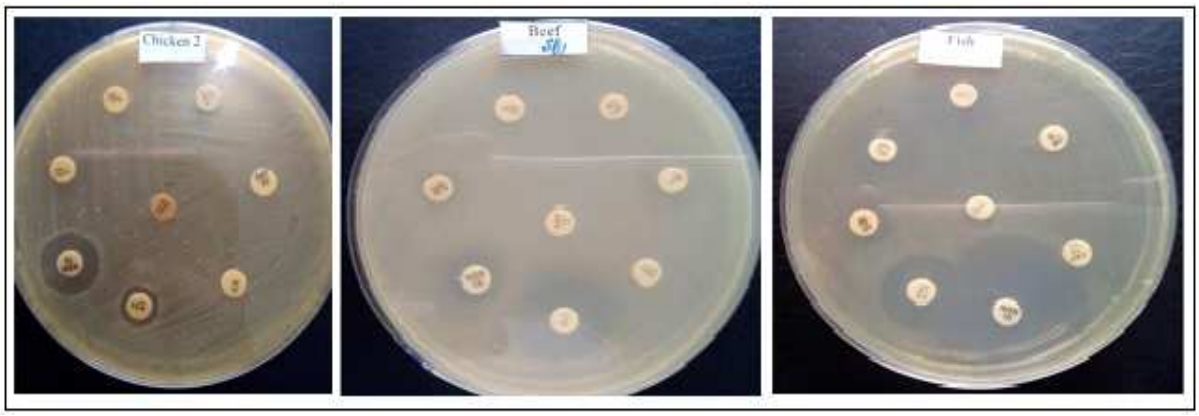

Fig. 12. Antibiotic sensitivity of *Acinetobacter species* isolates of beef, chicken meat and fish meat.

Conclusion

It can be concluded that *Acinetobacter* is a potential threat for transmitting the antibiotic resistance to the human from meat during handling process. Although the extensive cooking process can cause death of bacteria, but the problem may arises due to the consumption of half or under cooked meat.

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