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Bacteriological Characteristics, Antimicrobial Resistance Profile and Molecular Identification of *Acinetobacter* species Isolated From Meat of Different Sources in Egypt

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ABSTRACT

Although food is very important for human life, it may be life-threatening. Foodborne diseases are spreading worldwide through the increasing rate of fresh and undercooked food consumption. Foodborne pathogens include many types of bacterial species. This study was conducted to determine the prevalence of *Acinetobacter* species isolated from meat samples and their phenotypic characteristics, antimicrobial resistance profiles, and genotypic characteristics. A total of 110 samples, collected from chickens (n=50), beef (n=44), rabbits (n=10), and mutton (n=6), were examined bacteriologically. The suspected colonies were identified biochemically and then tested for antimicrobial resistance, biofilm formation, and hemolytic activity; identification was confirmed by Polymerase Chain Reaction (PCR) for the genes; *rpo*B, *tar*T, *fim*H, and *esp*A. Nine *Acinetobacter* species isolates (8.2%) were recovered. Fifty five samples resulted in the isolation of non-lactose fermenters with an incidence of 50%, 29 produced late lactose fermenters with an incidence of 26%. The rest of the samples showed no growth or non-lactose fermenters. On antibiogram, the isolates showed high resistance to ceftriaxone, imipenem, ceftazidime and ticarcillin/clavulanic acid in percentages of 89%, 77.8%, 66.7% and 66.7 %, respectively. While low resistance was found to sulfamethazole/ trimethoprim, doxycycline and amikacin in percentages of 44.4%, 33.3% and 11.1%, respectively. However, the isolates showed no resistance to ciprofloxacin. All the isolates were MDR with MDR_{index} (more than 0.5). Only one isolate was a weak biofilm producer but, no isolates produced hemolysis of the sheep RBCs. Genetically, 88.9% of the isolates expressed *tar*T and *fim*H genes, while only 5.6% of the isolates expressed *esp*A gene. It can be concluded that *Acinetobacter* species are to be considered when inspecting meat samples of different sources.

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INTRODUCTION

Acinetobacter is a Gram-negative, catalase-positive, oxidase-negative, strictly aerobic coccobacillus bacterium that arranges in pairs and exhibits twitching motility (**Rebic *et al*., 2018**). It causes one of the most critical hospital-acquired infections, especially in ICUs, leading to pneumonia, septicemia, meningitis, cystitis, and wound and skin infections **(Askari *et al*., 2019)**. Acinetobacters can be recognized in fresh, undercooked food and various foodstuffs as fruits, vegetables, raw milk and milk products **(Almasaudi, 2018)**, causing foodborne diseases **(Damaceno *et al*., 2015; Elbehiry *et al*., 2017)**.

Unhygienic measures and bad handling practices associated with meat processing of ready-to-eat products such as vegetables and fruits lead to cross-contamination with many harmful bacteria including *Acinetobacter* species **(Zhang *et al*., 2014; Askari *et al*., 2020)**. The utilization of antibiotics by animals has caused the generation of resistant bacteria which can be further disseminated to the environment through the food **(Phillips *et al*., 2004)**.

This study aimed to determine the prevalence of *Acinetobacter* species in the meat of different sources and determine the phenotypic characteristics, antimicrobial resistance profile, and virulence traits; and their genetic existence by PCR. The investigated

genes were *tar*T, type 1 fimbriae (*fim*H) and exopolysaccharide (*esp*A).

MATERIIALS AND METHODS

Sample collection:

A total of 110 samples were collected from chickens meat (n=50), beef (n=44), rabbit meat (n=10), and mutton (n=6) between June 2020 and March 2021. The sample sources were different butchers, supermarkets, hospitals' kitchens in Cairo and Qalubyia governorates belonging to 10 convenience store groups. All samples were examined for their sensory and physical properties such as odor, color, and texture. They were then collected in labeled sterile bags and transferred, while cold, immediately to the microbiology laboratory, Faculty of Veterinary Medicine, Cairo University.

Bacteriological examination of the samples:

The tested samples were initially inoculated into non-selective enrichment broth, selenite F-broth and incubated at 30˚C for 24 hr. **(Ruiz-Roldan *et al*., 2018)**. A Loopful of the inoculated broth was streaked onto MacConkey agar (Oxoid) and incubated at 30˚C for 48hr. A single colony form, late lactose and non-lactose fermenter colonies were streaked onto Leed *Acinetobacter* agar (LAM) (HI media) and incubated at 30˚C for 24hr. A typical pink colony on a purple background represents its morphology and shape on LAM (Fig. 1).

Identification of suspected *Acinetobacter* species:

Acinetobacter species isolates were identified based on colonial morphology, microscopical characteristics (Gram-negative rods to coccobacilli, with different sizes, usually arranged in groups and may appear violet, confusing with Gram-positive), and their biochemical testing; oxidase, catalase, TSI, motility, indole, methyl red, Voges-Proskauer, citrate utilization and urease test according to **Vos *et al*., (2011)**. Stock cultures were conserved in 20% sterile buffered glycerin at -80℃ for further studies.

Pure cultures of suspected *Acinetobacter* species were confirmed by PCR targeting the 16S rRNA gene and the virulence genes (*tra*T, *fim*H, and *eps*A) using species-specific primers (Table. 1). DNA extraction was done using PathoGene-spin™ DNA/RNA Extraction Kit following its manufactures' instructions. PCR conditions are shown in table (2).

Detection of phenotypic virulence traits:

Detection of the biofilm-forming ability of *Acinetobacter* species by Tissue culture plate method (TCP):

The ability to biofilm formation on abiotic surfaces was performed as described by (**Mussi *et al*., 2010**). A microplate reader was used to determine the biofilm formation at A_{640} nm and classified based on the level of their score, i.e., isolates with a score value of; over 0.55 were considered to be high biofilm producers, whereas those with less than 0.14 were considered as non-biofilm producers, and that of OD_{640}nm values falling between 0.14 and 0.55 were classified as moderate biofilm producers. Every test was conducted in triplicate. In this essay, the negative control wells contained sterile Brain Heart Infusion Broth (BHI), while positive controls contained *Staphylococcus epidermidis* ATCC 35984.

Fig.1: A: LAM of non-*Acinetobacter* growth, B: LAM of +ve *Acinetobacter* growth pink colony on purple background

Table 1: The oligonucleotides primer sequences used for molecular identification of *Acinetobacter* species isolates

Gene	Oligonucleotide sequences (5'-3')	Product size (bp)	Reference
16S rRNA (*rpo*B)	F: TAY CGY AAA GAY TTG AAA GAAG R: CMA CAC CYT TGT STM CCR TGA	397	Rafei, *et a*l., 2015
*tra*T	F: GGT GTG GTG CGA TGA GCA CAG R: CAC GGT TCA GCC ATC CCT GAG	290	Bahador, *et al*. 2013
*fim*H	F: TGC AGA ACG GAT AAG CCG TGG R: GCA GTC ACC TGC CCT CCG GTA	508	Bahador, *et al.* 2013
*eps*A	F: AGC AAG TGG TTA TGG AAT CG R: ACC AGA CTC ACC CAT TAC AT	451	Toledo-Arena, *et al.* 2001

Table 2: PCR Cycle conditions of *rpo*B, *fim*H, *eps*A, and *tra*T gene

Gene	Steps	Temperature	Time	Reference
*rpo*B	Initial denaturation	95°C	5 min	Rafei *et al*., (2015)
	35 Cycles of Denaturation Annealing Extension	 94°C 53°C 72°C	 45 s 45 s 45 s	
	Final extension	72°C	10 min	
*fim*H	Initial denaturation	94°C	4 min	Momtaz *et al*. (2015)
	34 cycles of denaturation Annealing Extension	 94ºC 56°C 72°C	 60 s 45s 60 s	
	Final extension	72°C	10 min	
*eps*A	Initial denaturation	95°C	1 min	Momtaz *et al*. (2015)
	30 Cycles of Denaturation Annealing Extension	 95°C 60°C 72°C	 30 s 60 s 60 s	
	Final extension	72°C	4 min	
*tra*T	Initial denaturation	94°C	4 min	Momtaz *et al*. (2015)
	34 Cycles of Denaturation Annealing Extension	 94°C 56°C 72°C	 60 s 45 s 60 s	
	Final extension	72°C	10 min	

Detection of the hemolytic activity of *Acinetobacter* species

The hemolytic activity of *Acinetobacter* species isolates was determined using brain heart infusion agar (LABM) supplemented with 5% sheep blood. The inoculated plates were incubated at 37°C for 24 hours. The hemolytic activity was documented as β-hemolysis, α-hemolysis, or γ-hemolysis. *Staphylococcus aureus* (ATCC25923) was used as a positive control **indicator strain (Tayabali *et al*., 2012).**

The antimicrobial resistance profile of *Acinetobacter* species isolates:

The disk diffusion method was employed using Mueller–Hinton agar (HiMedia Laboratories), according to Clinical and Laboratory Standards Institute guidelines **(CLSI, 2017)**. The used antimicrobial disks were sulfamethazole/trimethoprim (COT 23.75µg /1.25µg), doxycycline (DO 5 µg), ceftriaxone (CTR 30 µg), ceftazidime (CTX 30 µg), ciprofloxacin (CIP 5 µg), imipenem (IPM 10 µg), amikacin (AK 30 µg), and ticarcillin/clavulanic acid (TIM 75/10 µg). The MDR_{index} was calculated by the equation a/ (bxc), where 'a' was the aggregate antibiotic resistance score of all isolates from the sample, 'b' was the number of antibiotics used (n=8 via this study) and 'c' was the number of isolates originated from the sample (**Krumpeman, 1983**). The criteria followed the standardized international terminology for defining multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan drug-resistant (PDR), which was initially created through a joint initiative by the European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention (**Magiorakos *et al*., 2012**).

RESULTS

From the examined samples, 9 *Acinetobacter* species isolates (8.2%) were recovered from chicken meat (n=4), beef (n=3), and rabbit meat (n=2) with percentages of 3.6%, 2.7% and 1.8%, respectively while, no *Acinetobacter* species could be isolated from mutton. Fifty-five of the examined samples were non-lactose fermenters with an incidence of 50%, 29 produced late lactose fermentation with an incidence of 26% and the rest of the samples showed no growth (Fig.2 and table. 3). All isolates were oxidase negative, catalase-positive, nitrate reduction negative, indole

negative, non-motile, glucose fermentation positive, sucrose negative. Concerning lactose fermentation: most isolates were negative and some were positive.

Fig.2 : A: Non-lactose fermenter on MacConkey agar, B: late lactose fermenter on MacConkey agar

Table 3: Prevalence of *Acinetobacter* species isolates from the examined samples

Type of samples		No. of examined samples	No. of non-lactose fermenter sample After 24 hrs		No. of late lactose fermenter Sample After 48 hrs	
			No.	%	No.	%
Chicken meat	Local raw White	19	13	26	4	8
	Local raw red	12	5	10	4	8
	Imported	9	4	8	1	2
	Ready- to- eat	10	8	16	0	0
	Total	50	30	60	9	18
Beef meat	Local raw buffalo	13	2	5	11	25
	Cows	12	9	20	3	7
	Imported	9	2	5	1	2
	Ready- to- eat	10	7	16	0	0
	Total	44	20	46	15	34
Rabbit meat		10	5	50	4	40
Mutton		6	0	0	1	17
Total		110	55	50	29	26

* The percentage was calculated according to the total number of positive samples.

Antimicrobial resistance profile of *Acinetobacter* species isolates:

All *Acinetobacter* species isolates were tested for their susceptibility against 8 antibiotics, representing 6 different classes. A high resistance pattern was noticed in all *Acinetobacter* isolates to ceftriaxone, imipenem, ceftazidime and ticarcillin/clavulanic in 89% and 78%, 67% and 67%, respectively. Meanwhile, low resistance was found to sulfamethazole/trimethoprim, doxycycline and amikacin in a percentages of 44.4%, 33.3% and 11%, respectively. However, no resistance to ciprofloxacin was detected. All isolates were MDR with MDR index of more than 0.5.

Biofilm forming ability of *Acinetobacter* species by tissue culture plate method (TCP):

The biofilm *index* was calculated for nine *Acinetobacter* isolates. It appeared that one isolate was a weak biofilm producer (11%) while all other isolates were non-biofilm producers (89%) (Table. 4).

Table 4: Biofilm Formation Assay by TCP of *Acinetobacter* species isolates:

Code	1st OD reading	2nd OD reading	3rd OD Reading	Mean OD± standard deviation	Biofilm status
Negative Control	0.1272	0.1715	0.1073	0.1353± 0.033	
1	0.1513	0.1394	0.1095	0.133± 0.022	Non-adherent
2	0.1012	0.0747	0.4024	0.193± 0.182	Non-adherent
3	0.1016	0.0739	0.1525	0.109± 0.040	Non-adherent
4	0.1212	0.0753	0.0842	0.094± 0.024	Non-adherent
5	0.0745	0.1795	0.0868	0.114± 0.057	Non-adherent
6	0.1556	0.1151	0.1701	0.147± 0.029	Non - adherent
7	0.0757	0.1389	0.0973	0.104± 0.032	Non-adherent
8	0.0705	1.2115	0.3071	0.530± 0.602	Weakly-adherent
9	0.0733	0.1971	0.1971	0.156± 0.071	Non - adherent

OD: optical density measured by ELISA reader at 640 nm.

The hemolytic activity of *Acinetobacter* isolates:

No *Acinetobacter* isolate showed any hemolytic activity (Fig. 3).

Fig. 3: Non-hemolytic *Acinetobacter* spp on blood agar

The Antibiotic sensitivity testing:

The antibiogram results revealed that *Acinetobacter* species in chicken was more resistant to DO, CTR, CTX, IPM, and TIM while, that of beef were resistant to COT, CTR, CTX, IPM, and TIM while, the rabbit-originated isolates were resistant to COT, CTR, CTX, and IPM (Fig. 4 and table. 5).

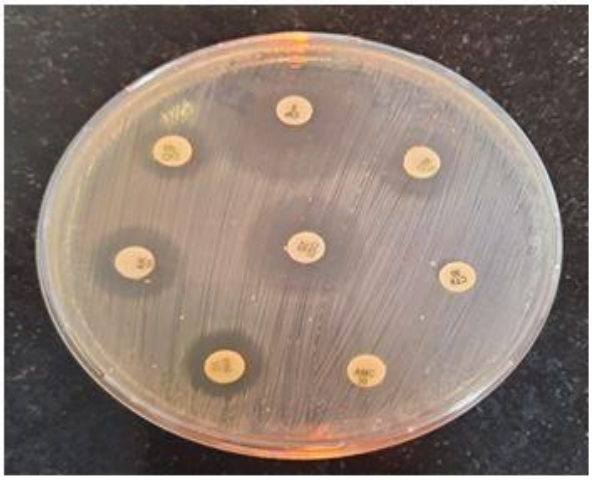

Fig. 4: Antibiotic sensitivity testing result

Table 5: Antibiotic sensitivity testing of each *Acinetobacter* species isolates

	Anti-microbial agents	COT	DO	CTR	CTX	CIP	IPM	AK	TIM
1	Chicken meat	S	R	R	I	S	R	S	I
2	Chicken meat	R	I	R	R	S	R	R	R
3	Chicken meat	S	S	R	R	S	R	S	R
4	Chicken meat	S	I	R	R	S	R	S	R
5	Beef	R	R	I	R	S	I	S	R
6	Beef	R	S	R	I	S	R	I	R
7	Beef	S	S	R	R	S	R	S	R
8	Rabbit	I	S	R	I	S	I	S	I
9	Rabbit	R	R	R	R	S	R	I	S

Molecular screening of the virulence genes of *Acinetobacter* species:

All the tested isolates were positive for 16S rRNA gene (*rpo*B) (100%) as shown in Fig. 5), while eight out of nine *Acinetobacter* isolates (89%) harbored *tar*T and *fim*H genes (Fig. 6 and 7), and, five isolates contained the *esp*A gene (55.6%) (Fig. 8).

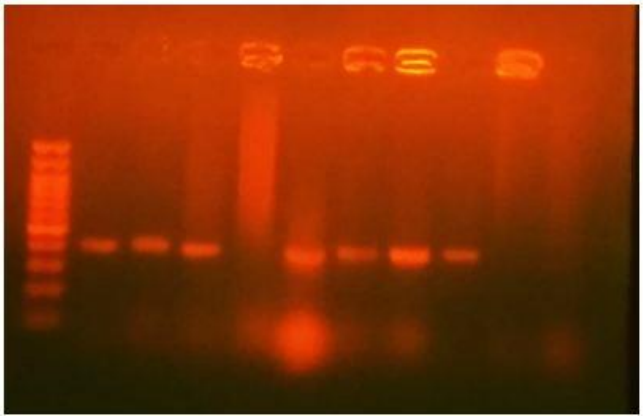

Fig.5: Agarose gel electrophoresis showing amplification of 397 bp fragment for 16*S* rRNA gene of the *Acinetobacter* species isolates. Lane 1: 1 kb Ladder, Lanes(2,3,4,6,7,8,9): positive *Acinetobacter* species, Lanes(5,10): negative for *Acinetobacter* species.

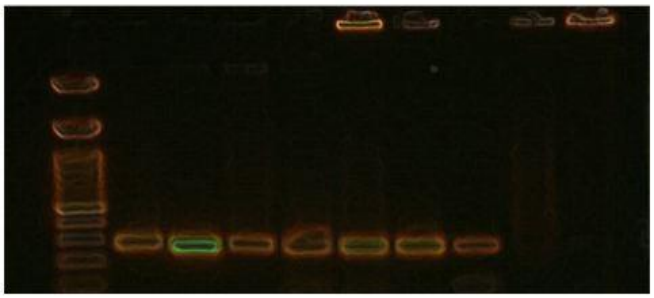

Fig. 6: Agarose gel electrophoresis showing amplification of 508 bp fragment for *fim*H gene of *Acinetobacter* species isolates. Lane 1: 1 kb Ladder, Lanes (2, 3, 4, 5, 6, 7, 8): positive for *Acinetobacter* species at 870bp.

Fig.7: Agarose gel electrophoresis showing amplification of 290 bp fragment for *tra*T gene using specific primer. Lane 1: 1 kb Ladder, Lanes (2,3,4,5,6,7,8): positive *Acinetobacter* species.

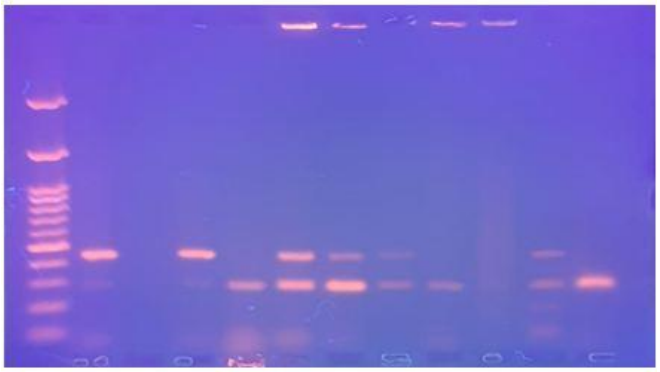

Fig.8; Agarose gel electrophoresis showing amplification of 451 bp fragment for *eps*A gene using specific primer. Lane 1: 1 kb Ladder, Lanes (2,4,6,7,8,11): positive for *Acinetobacter* species.

DISCUSSION

Acinetobacter species are highly dispersed that can be recovered from food samples such as fish, meat, cheese, milk, and vegetables. This is in addition to different environmental sources such as activated sludge, sewage, dumpsites, raw wastewater, and hydrocarbon-contaminated areas (**Doughari *et al*., 2011**). In the present study, the incidence of *Acinetobacter* species was 8.2% and that agrees with **Marí-Almirall *et al*., (2019),** who recovered *Acinetobacter* species (8.6%) from meat samples. Incidences of *Acinetobacter* species isolates from chicken meat, beef and rabbit meat were 3.6%, 2.7% and 1.8%, respectively, while **Ahmad *et al*., (2018)** could isolate *Acinetobacter* (26.6% and 23.3%) from chicken meat and beef, respectively.

In the current study, *Acinetobacter* species isolates showed high resistance to cephalosporins and carbapenem, which might be due to their intrinsic resistance to cephalosporins. This result was in accordance with those of **Marí-Almirall *et al*., (2019)**, whose isolates; from different meat sources; showed high resistance to cephalosporins. On the contrary, *Acinetobacter* species isolates were less resistant to tetracyclines, aminoglycosides and sulpha drugs; these results correspond to **Lupo *et al*., (2014)** who reported a few resistant isolates to tetracycline and **Elnar *et al*., (2020)** who observed the susceptibility of *Acinetobacter* species to a broad range of antimicrobials including tetracyclines.

All the recovered strains in the current study were MDR. **Wareth *et al*., (2019)** reported that only one *Acinetobacter* isolate was MDR. The antimicrobial-resistant bacteria in meat samples may be attributed to the extensive use of antimicrobials for treatment, prevention and control of diseases and as growth promoters in food-producing animals; this harmonizes with the study of **Askari *et al*., (2019)**.

It was surprising that all *Acinetobacter* species isolates were non-hemolytic. However, all the types of hemolytic activity had been identified in the studies of; **Tayabali *et al*., (2012), who** mentioned that from all the tested isolates, there were 2 β-hemolytic isolates, 3 α-hemolytic activity isolates and 2 γ-hemolytic activity; **Dahdouh *et al*., (2016)** who reported that 46.7% of *A. baumannii* isolates showed α-hemolysis on blood agar while one isolate showed β-hemolysis, 80% showed γ-hemolysis. From the tested *Acinetobacter* species isolates, only one showed weak biofilm production. In contrast, 85.6% of *A. baumannii* isolates tested by **Dahdouh *et al*., (2016)** showed strong biofilm formations, 11.1% showed weak formations and 3.3% showed no biofilm formation. In contrast, **Zeighami *et al*.**, (**2019)** mentioned that all *A. baumannii* isolates could produce either moderate or strong biofilm.

The prevalence of the virulence genes, *tar*T and *fim* H gene was 89%, for each while, for *esp*A gene, the prevalence was 55.6%; this result was a little different from **Tavakol *et al*., (2018)** who mentioned that *the fim*H gene was commonly detected among his isolates while *tra*T (serum resistance) non-adhesive virulence factor were low. Also, **Adewoyin, (2020)** reported that *traT* was detected in few *Acinetobacter* isolates.

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CONCLUSION

To our knowledge, we report the first identification of *Acinetobacter* species from the meat of different sources in Egypt. Despite the fact that the extensive cooking process can kill the bacteria, the problem may arise due to half or undercooked meat consumption. That is considered a potential threat for transmitting antibiotic resistance from meat to humans. Further studies should be applied to define *Acinetobacter* species and discover the genetic differences between animal and human isolates.

Declaration of Conflicting Interests

The authors revealed that there is no potential conflicts of interest.

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