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# IDENTIFICATION OF ANTIBIOTIC RESISTANCE GENES FOR *CAMPYLOBACTER FETUS* ISOLATED FROM INFERTILE AND ABORTED BOVINES IN SOME EGYPTIAN GOVERNORATES

By

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## ABSTRACT

In this study, 266 samples including 81 preputial swabs, 34 semen samples from bulls and 118 vaginal swabs, 16 uterine discharges and 17 aborted foeti from dairy cows suffering from infertility and abortion were randomly collected from January - December 2016 from some governorates in Egypt.The antimicrobial susceptibilities were applied against 12 antimicrobial agents.All the *Campylobacter* isolates were tested for the presence of *AphA -3-1*and *blaOXA-61* genes. The results showed that *Campylobacter* isolates were 52(19.55%) of the total samples (n= 266) with prevalence of *Campylobacter fetus subspecies venerealis (CFV*), 32 (61.54%),followed by*Campylobacter fetus subspecies fetus* (*CFF*),20 (38.64%). The highest incidence of *Campylobacter* spp. was (37.5%) in uterine discharges, followed by aborted foeti (23.53%) then in vaginal swabs (22.88%) and in preputial swabs (18.52%) respectively. The highest incidence of *CFV* was in uterine discharges (25%), aborted foeti (17.65%), and vaginal swabs (13.56%) and in preputial swabs (11.11%) respectively. Out of the *Campylobacter* isolates, 23 (44.23%) showed the right band (701 bp) that which denotes phenotypic resistance to erythromycin is contained *AphA* gene. Twelve (23.07%) out of 52 *Campylobacter* isolates showed the right band (372 bp) which is contained the *blaOXA- 61* gene that denotes phenotypic resistance to ampicillin. Finally, we have concluded that the existence of antimicrobial resistant genes in *Campylobacter* spp. will reduce the efficacy of several classes of antimicrobials commonly used in the treatment resulting in high morbidity and mortality rates associated with subsequent economic losses due to the use of ineffective antimicrobials.

**Key words:**

*Campylobacter fetus subspecies venerealis*, *Campylobacter fetus subspecies fetus*, antimicrobial resistance genes.

## INTRODUCTION

Bovine venereal campylobacteriosis is caused by *Campylobacter fetus subsp. venerealis* and *Campylobacter fetus subsp. fetus*. The disease can be economically important when present in cattle herds, causing poor reproductive performance, embryo mortality and abortion **(Truyers, *et al.* 2014).** *C. fetus subsp. venerealis* (*Cfv*) is a sexual seasonally transmitted disease which has been isolated from the reproductive tract of cattle and buffaloes from internal organs of aborted foetuses **(Campero, *et al.* 2005)**. *C. fetus subsp. fetus* (*Cff*) is transmitted orally and colonized the intestines of cattle and sheep, causing enteritis and abortion mostly in sheep and sporadically in cattle **(Garcia and Eaglesome, 1983)**. In heifers and cows the bacteria spread to the uterus and oviducts resulting in endometritis and salpingitis. Infection does not affect conception but will typically result in early embryonic death and thus delayed return to estrus **(Schulze, *et al.* 2006).** Abortions can occur at any time but are most commonly detected at 4 to 6 months of gestation. Most cows will self-recover and conceive within 3 to 6 months' post-infection and the acquired immunity lasts for several years **(Mshelia, *et al*, 2007)**. In bulls the infection is asymptomatic and bacteria can colonize the crypts of the preputial epithelium, and as bulls age, the size and number of these crypts increase allowing persistence of infection, and these referred to as chronic carrier status which makes both diagnosis and treatment more difficult **(Taylor, 2002)**. Although *Campylobacter* infections are usually self-limiting and do not require antibiotic treatment, however, in severe cases of prolonged enteritis and septicemia, antimicrobial treatment is often needed **(Engberg *et al.* 2001 and Guevremont *et al.* 2006)**. Macrolides and fluoroquinolones are commonly prescribed for campylobacteriosis; however, the resistance to these and to the other antibiotics also occurs **(Moore *et al.* 2006)**. The current increasing trend in antibiotic usage holds a serious danger for the future and, therefore calls, for alternative plans to safeguard future livestock production, food security and human health. This becomes more imperative considering emerging resistance against tetracycline's and fluoroquinolones, the foremost remedies for livestock diseases in most developing countries **(Roz´ynek *et al.* 2010)**. Resistance to antibiotics has continued to increase, placing future animal and human disease management in real danger. The developing countries are characterized by widespread indiscriminate antibiotic use and in which 'third-generation' antibiotics are not readily available or affordable are the worst affected **(Wayne, 2010)**. The use of antibiotics in livestock management has been reported as one of the factors responsible for the development of resistant bacteria and

dissemination from livestock strains to human **(Murray ,1998)**.This is because antimicrobials used in the treatments of infected animals are in most cases of the same class of antimicrobials as those used in human medicine and may co-select for antimicrobial resistance in bacteria such as, enterococci during consumption of animal food **(Guardabassi, *et al.* 2004)**. Apart from being one of the primary causes of nosocomial infection, enterococci are also known as a reservoir of antimicrobial resistance genes **(Huycke, *et al.*1998)**.The aim of this study was to investigate the prevalence, antimicrobial resistance, and genetic determinants of resistance of *Campylobacter* isolated from infertile and aborted cows and bulls in Egyptian farms.

## MATERIAL AND METHODS

### 1- Sampling:

In this study, 266 samples from cattle and buffaloes; including 81 preputial swabs and 34 semen samples from bulls, 118 vaginal swabs, 16 uterine discharges and 17 aborted foeti from dairy cows suffering from infertility and abortion were randomly collected from January - December 2016 from some governorates in Egypt. All samples were aseptically taken in sterile plastic bags and were immediately transported to the laboratory in a cooler with ice packs and processed immediately upon arrival for isolation of *Campylobacter.*

**Table (1):** Samples collected from different bovine farms in Egypt.

| Type of samples | Animal | | No. of samples |
|---|---|---|---|
| | cattle | buffaloes | |
| Preputial swabs | 60 | 21 | 81 |
| Semen samples | 20 | 14 | 34 |
| Vaginal swabs | 90 | 28 | 118 |
| Uterine discharges | 10 | 6 | 16 |
| Aborted foeti | 12 | 5 | 17 |
| Total | 192 | 74 | 266 |

### 2- Isolation and identification of *Campylobacter* species:

Samples were swabbed onto Thioglycolate broth, they were incubated at 37°C for 48 h in an anaerobic jar with a gas generating sachet (Oxoid - Campy Gen TM) to produce a microaerophilic atmospheric condition (5% $O_2$, 10% $CO_2$ and 85% $N_2$) for the growth of *Campylobacter* spp **(Avrain *et al.* 2003)**. Wet *Campylobacter* preparations were primarily identified under phase contrast microscope for detection of the characteristic motility and morphological character

according to **(Smibert, 1984)**. A loopful of enrichment broth was plated on modified Karmali agar plates (Oxoid) with 10% sheep RBCs and incubated in microaerophilic atmosphere at 37 ºC/48 hrs. **(Persson and Olsen, 2005)**. Suspected colonies of *Campylobacter* isolates were further subcultured and identified by biochemical tests described by **(Frost *et al* .1998)** including growth at 25ºC, at 37ºC and at 43ºC, growth in the presence of 3.5% NaCl and 1% glycine, motility, catalase test, oxidase test, $H_2S$ production on triple sugar iron agar (TSI) agar, sodium hippurate hydrolysis and susceptibility to nalidixic acid and cephalothin (Table 5). Identified colonies were stored at -70 in nutrient broth with 15% glycerol until examined by antibiotic susceptibility testing **(Sheppard *et al* .2009)**.

**3- Antibiotic Susceptibility testing:**

The antimicrobial susceptibilities were applied to 12 antimicrobial agents (ampicillin, erythromycin, trimethoprim, sulphamethoxazole, amoxicillin, clorithrancin, streptomycin, gentamicin, ofloxacin, ciprofloxacin, levofloxacin, and ceftriaxone) with Mueller-Hinton agar (Oxoid) plates based on the Clinical and Laboratory Standard guidelines **(CLSI 2008)**. The plates were then incubated at 37°C for 24 h in an anaerobic jar with a gas-generating sachet to enable the *Campylobacter* spp. to grow, and antimicrobial-free agar plates were included as a control for normal growth **(ROSCO 2007)**.

**4- Extraction of *DNA:***

*Campylobacter* isolates were cultured on 5% horse blood agar plates and incubated at 42°C for 48 h in an anaerobic jar. After sufficient growth was obtained, the bacterial cells were harvested from the plates and placed in Eppendorf tubes containing 200 µl of sterile milli Q water. The suspensions were placed on a heating block for 8 min at a temperature of 98°C. These were then centrifuged at 17000 xg for 5 min and the supernatants were transferred into fresh Eppendorf tubes to serve as a DNA template for subsequent (PCR) **(Kabir *et al.* 2011)**.

**Table (2):** Oligonucleotide primers used for *Campylobacter fetus.*

| Target gene | Primer sequence | Band size bp |
|---|---|---|
| ***glnA* (*CFV*)** | **GAT GGT AGT TCT ATA GAC GC**<br>**CTT CCG TTA TCT CCA TAA AGC** | **585 bp** |
| ***glyA* (*CFF*)** | **GAT AAA ATA CTT GGT ATG GAT C**<br>**CCC TCT GTT TAT TAA GAC TTC** | **290 bp** |

**5- Speciation and identification of PCR:**

Primers targeting the *Campylobacter* genus-specific 23S rRNA gene and species-specific regions of *C.f. venerealis* and *C.f. fetus* were used mixture containing 5 µl of PCR buffer, each of oligonucleotide primers of Taq polymerase (Promega) including 2 µl DNA template (Table 2). The volume was adjusted with sterile distilled water to give 25 µl. The amplification was carried out using 30 cycles of denaturation at 95°C 30 s, annealing at various temperatures for 30 s and extension at 72°C for 30 s, and ending with final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis using a 1.5% agarose gel, containing ethidium bromide.

**6- Detection of antibiotic resistant genes by using PCR:**

All the *Campylobacter* isolates were tested for the presence of *AphA-3-1*, and *blaOXA*-61 genes. A multiplex PCR was carried out using this amplification protocol: 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 1 min (Table 3 and 4). Electrophoreses on a 1·5% agarose gel containing ethidium bromide in a 19 mTAE buffer. The lengths of the various amplicons were determined by comparing them with 1000 bp ladder **(Obeng *et al*, 2012)**.

**Table (3):** Antimicrobial resistance genes in *Campylobacter* isolates.

| Antibiotic | MDR gene | Primers | Band size bp | Annealing temperature |
|---|---|---|---|---|
| **Ampicillin** | ***Bla*$_{oxa-61}$** | **F: AGAGTATAATACAAGCG R: TAGTGAGTTGTCAAGCC** | **372** | **54 C** |
| **Erythromycin** | ***AphA-3-1*** | **F: TGCGTAAAAGATACGGAAG R: CAATCAGGCTTGATCCCC** | **701** | **54 C** |

**Table (4):** Components of the PCR reaction mixture, for amplification of the antibiotic resistance genes in *Campylobacter* species isolated from bovine.

| | |
|---|---|
| **PCR water** | **14 ul** |
| **5X PCR buffer** | **4 ul** |
| **2 mmol $l^{-1}$ DNTPS** | **2 ul** |
| **Each primers (25 pmol )** | **1 ul (2)** |
| **Promega Taq polymerase (1 units/ul) Just use 1 unit** | **1 ul** |
| **DNA template** | **2 ul** |

**7- Statistical analysis:**

Comparisons of association between phenotypic resistance and resistance genes in *Campylobacter* from bovine isolates were performed using Chi-squared exact test (Fisher's exact two tail test). Statistical significance was set at a P value of <0·05.

## RESULTS

**Table (5):** Prevalence of the characterized *C.fetus* isolated from bovine samples.

| Type of samples | No. of samples | No. of positive samples | % | *Campylobacter* isolates | | | |
|---|---|---|---|---|---|---|---|
| | | | | *CFV** | | *CFF*** | |
| | | | | No. | % | No. | % |
| **Preputial swabs** | **81** | **15** | **18.52%** | **9** | **11.11%** | **6** | **7.41%** |
| **Semen samples** | **34** | **-** | **-** | **-** | **-** | **-** | **-** |
| **Vaginal swabs** | **118** | **27** | **22.88%** | **16** | **13.56%** | **11** | **9.32%** |
| **Uterine discharges** | **16** | **6** | **37.5%** | **4** | **25%** | **2** | **12.5%** |
| **Aborted foeti** | **17** | **4** | **23.53%** | **3** | **17.65%** | **1** | **5.88%** |
| **Total** | **266** | **52** | **19.55%** | **32** | **61.54%** | **20** | **38.46%** |

***CFV: C.f.ss. venerealis** ***CFF: C.f.ss. fetus**

**Table (6):** Biochemical characters of isolates of C.fetus in bovine samples.

| Biochemical character | CFV | CFF |
|---|---|---|
| Oxidase | + | + |
| Catalase | + | + |
| Nitrate reduction | + | + |
| Urease | - | - |
| Hippurate hydrolysis | - | - |
| Growth at: | | |
| 25◦C | - | + |
| 37◦C | + | + |
| 43◦C | - | - |
| Growth at 1% glycine | - | + |
| Growth at 3.5% NaCl | + | - |
| Susceptibility to: | | |
| Nalidixic acid | S | S |
| Cephalothin | R | R |

**+ve: positive -ve: negative S: sensitive R: resistance**

**Table (7):** Percentage of resistance against different antimicrobial agents among *Campylobacter i*solates**.**

| Antibiotic name | *CFV* | | *CFF* | |
|---|---|---|---|---|
| | *Susceptibility* | *%* | *Susceptibility* | *%* |
| Ampicillin | R | 80%, | R | 81.7%, |
| Erythromycin | R | 77%, | R | 79.8%, |
| Trimethoprim | R | 75% | R | 76.1% |
| Sulphamethoxazole | R | 70% | R | 71% |
| Ceftriaxone | R | 62% | R | 61% |
| Amoxicillin | R | 55% | + | 45% |
| Clorithrancin | (+) | 47% | R | 55% |
| Streptomycin | (++) | 60% | (++) | 55% |
| Gentamycin | (++) | 45% | (++) | 44% |
| Ofloxacin | (++) | 43% | (+++) | 42% |
| Ciprofloxacin | (+++) | 40% | (+++) | 41% |
| Levofloxacin | (+++) | 35% | (+++) | 37% |

***R: Resistance S: Sensitive ++: Moderate sensitive +++: Highly sensitive***

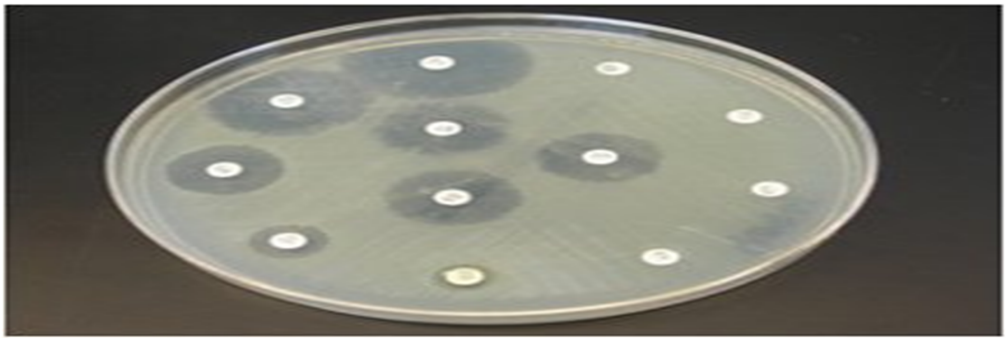

**Fig.(1):** Show resistance against different antimicrobial agents among Campylobacter isolates.

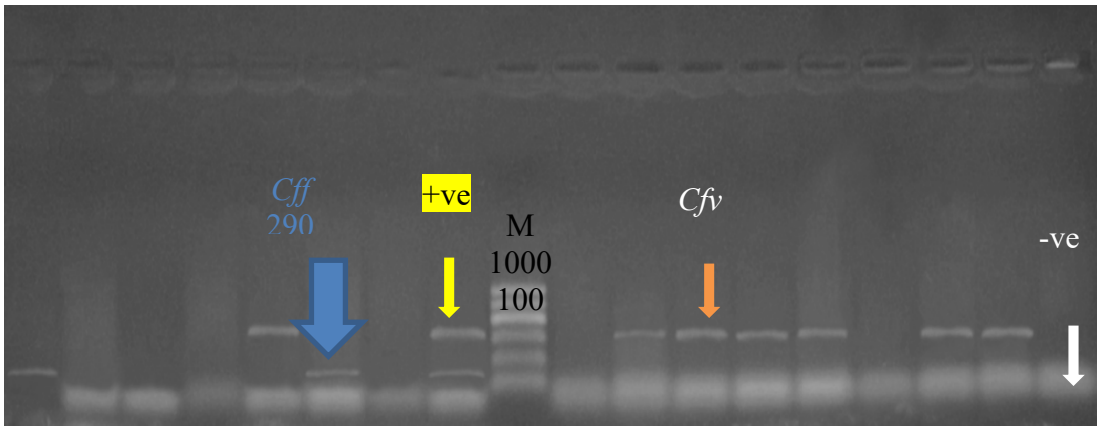


**Fig. (2):**Agarose gel electrophoresis of PCR for *Campylobacter* spp. LaneM: 1000 bp ladders. +ve= positive-ve=negative.*Campylobacter* isolates yielded the genus specific (16S *rRNA*) *Cfv* 585 bp Lane:5, 9,10,11,12, 14, and 15. *Cff* 270 bp Lane:1, 6.

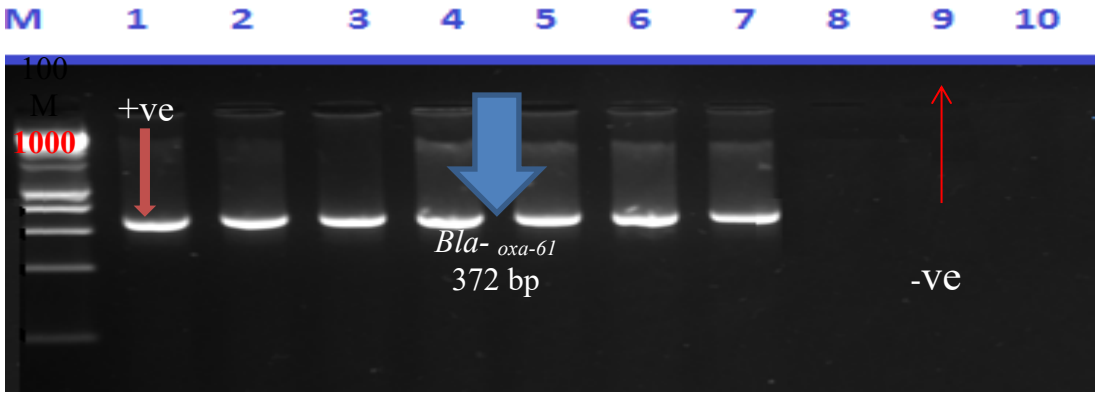


**Fig.( 3):** Agarose gel electrophoresis of PCR for Antimicrobial resistance genes in *Campylobacter* isolates. LaneM: 1000 bp ladders. +ve= positive Lane: 1, 2, -ve= negative Lane: 8, 9 & 10. *Bla-* $_{oxa-61}$ at 372 bp Lane: 3, 4, 5, 6 & 7.

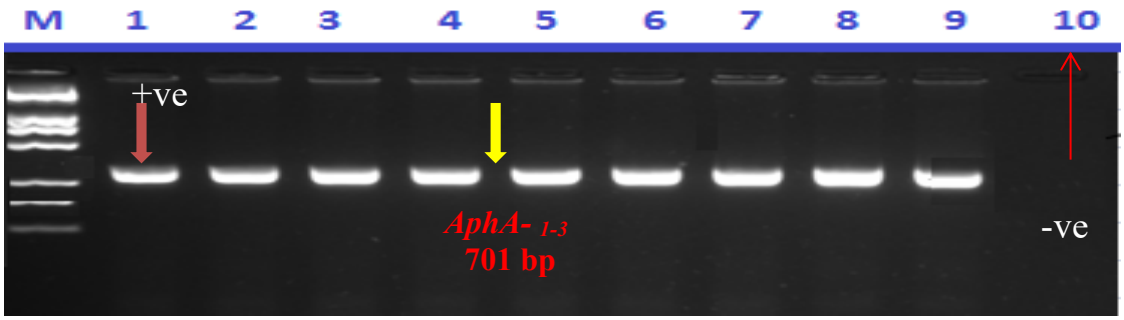


**Fig. (4):** Agarose gel electrophoresis of PCR for Antimicrobial resistance genes in *Campylobacter* isolates. Lane M: 1000 bp ladders.+ve= positive Lane:1,2,-ve= negative Lane: 10. *AphA-* $_{1\text{-}3}$ at 701 bp Lane: 3, 4, 5, 6, 7, 8 & 9.

## DISCUSSION

The results of this study showed that, the overall *Campylobacter* isolates were 52(19.55%) from the different samples of bovine where *CFV* was the most prevalent species 32 (61.54%) followed by *CFF* 20 (38.64%), as illustrated in (Table 5) and Fig. (2). Recorded data revealed the high incidence of *Campylobacter* isolates in uterine discharges (37.5%) followed by aborted foeti (23.53%) then in vaginal swabs (22.88%), and in the preputial swabs (18.52%). The high incidence of *CFV* was in uterine discharges (25%), aborted foeti (17.65%), in vaginal swabs (13.56%) and in preputial swabs (11.11%). These incidences agree with that recorded by **Atanssova and Ring (1998) and Bi, *et al.* (2008).** Also, the high percentage of *CFF* was in uterine discharges (12.5%), followed by vaginal swabs (9.32%); then preputial swabs 7.41% and low incidence in aborted foeti (5.88%). These findings agreed with **Ono and Yamamoto, (1999) and Sahin, (2003).** Since the introduction of antibiotic therapy, vast numbers of antibiotic-resistant bacteria has developed throughout the world. This acquired resistance is primarily a direct result of many years of underuse, misuse and overuse of antibiotics by humans (**Lawes, *et a*l 2012).** Incorrect usage and exposure to antibiotics increases the risk of the bacteria acquiring resistance to a specific antibiotic. Antibiotic growth promoters (AGPs) are still used intensively in Egyptian livestock industry and therefore contribute to the increase in resistance patterns of certain antibiotics **(Nauta, *et al.* 2005**). Antibiotic susceptibility testing was carried out for ampicillin, erythromycin, trimethoprim, sulphamethoxazole, amoxicillin, clorithrancin, streptomycin, gentamicin, ofloxacin, ciprofloxacin, levofloxacin, and ceftriaxone) according to the disk diffusion method for monitoring the prevalence of antibiotic resistant *CFV and CFF* (**Moore *et al.***

**2006).**The percentages of resistant *Campylobacter strains* (n = 52) of bovine samples were from 81.7%, 79.8%, 77.9%, 76.1%, till 35% (Table 7) and Fig. (1). the resistance prevalence is very high and is suggestive that previous exposure of the bacterial isolates to antibiotics is the main reason of the high resistance levels. These findings are consistent with published data from clinical Campylobacter isolates which show high levels of antimicrobial resistance **(Sanchez-Gonzalez, 2011 and Strachan and Forbes, 2010)**. Antibiotic resistance to ampicillin, erythromycin, trimethoprim,sulphamethoxazole and ceftriaxone showed resistance for both *C. fetus*, but *Cff* was sensitive to amoxicillin and resistance to clorithrancin and erythromycin. This finding is consistent with previous studies, in which associations were found between particular *Campylobacter* species which is a putative mechanism of resistance to antibiotics **(Endtz, *et al.* 1991 and Helen, *et al.* 2013)**.Twelve (23.07%) out of 52 *Campylobacter* isolates showed right band (372 bp) which is contained in the *blaOXA-61* gene phonotypical resistance to ampicillin Fig. (3). Out of *Campylobacter* isolates, 23 (44.23%) showed right band (701 bp) contained in *AphA*1-3 gene have revealed phonotypic resistance to erythromycin Fig. (4). Ampicillin resistance is chromosomally encoded **(Zhu, *et a*l. 2006)**. A single nucleotide mutation (G→T transversion) upstream of *blaOXA-61* was identified in the ampicillin-resistant derivative of *Campylobacter* species **(Zeng, *et al.* 2014**). The results of the phenotypic and genetic analyses of antimicrobial susceptibility were fully concordant associated with the presence of the *blaOXA-61* gene carried on the chromosome. Our results are similar to those described by **Allen, *et al.* (2008) and Obeng *et al.* (2012**). Macrolide antibiotics that inhibit bacterial protein synthesis including erythromycin are the treatment of choice for campylobacteriosis **(Guerrant, *et al.* 2002)**. Other members of this class of antibiotics include clarithromycin and azithromycin, are approved for veterinary use only. The main mechanisms of resistance to macrolides in *Campylobacter* are target modification, efflux and altered membrane permeability **(Velázquez, *et al.* 1995).** Our results suggest that, the resistant strains are persisting environmental isolates that have been acquired by the differential livestock species. Furthermore, the different treatment practices in bovines have resulted in differences in resistance profiles in *Campylobacter* isolates **(Adesokan, *et al.* 2015).** The results encourage the prudent use of antimicrobials by clinicians, pharmaceutical companies in order to decrease the public health risk associated with the potential spread of antimicrobial resistance bacteria or their genes from food producing animal to human **(Hershberger *et al.* 2005 and Marshall**

**and Levy 2011)**. Finally, we concluded that, the development of antimicrobial resistance in treatment of campylobacteriosis will reduce the efficacy of several classes of antimicrobials commonly used in the treatment resulting in high morbidity and mortality rates associated with subsequent economic losses due to the use of ineffective antimicrobials.

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# التعرف على الجينات المقاومة للمضادات الحيوية لميكروبات الكامبيلوباكتر الجنينى المعزولة من الماشية المصابة بقلة الخصوبة و الاجهاض في بعض المحافظات المصرية

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## الملخص العـــــربى

في هذه الدراسة تم تجميع عدد 266 عينة من الابقاروالجاموس تحتوى على عدد 81 مسحة جرابية وعدد 34 عينة من السائل المنوي من الثيران وعدد 118 مسحة مهبلية وعدد 16 عينة من افرازات الرحمية وعدد 17 اجنة مجهضة من الأبقارو الجاموس التي تعاني من قلة الخصوبة والإجهاض، تم جمعها فى الفترة من من يناير إلى ديسمبر 2016 من بعض المحافظات في مصر. تم تطبيق اختبار الحساسية المضادة للميكروبات لعدد 12مضاد حيوى تم اختبار جميع معزلات الكامبيلوباكتر الجنينى المعزولة فى الدراسة لوجود الجينات

*المقاومة للمضادات الحيوية. $AphA_{1-3}$ و* ***bla*** *OXA-61* **gene**

وأظهرت النتائج أن اجمالى عدد معزولات الكلى الكامبيلوباكتر الجنينى 52معزولة بنسبة (19.55٪) من مجموع العينات (ن = 266) كان عدد الكامبيلوباكتر الجنينى الضارى 32 معزولة بنسبة (61.54٪)، يليها الكامبيلوباكتر الجنينى المعوى عدد 20معزولة بنسبة (38.64٪). وكانت أعلى نسبة للعزل من الافرازات الرحمية هى (37.5٪)، تليها الاجنة المجهضة بنسبة (23.53٪) ثم المسحات المهبلية (22.88٪) واقلها في المسحات الجرابية للطلائق بنسبة (18.52٪) على التوالي وكانت أعلى نسبة لتواجد ميكروبات الكامبيلوباكتر الجنينى الضارى فى الافرازات الرحمية بنسبة (25٪)، وفى الاجنة المجهضة بنسبة (17.65٪) ثم المسحات المهبلية بنسبة (13.56٪) والمسحات الجرابية بنسبة (11.11٪) على التوالي.

وأظهرت معزولات الكامبيلوباكتر أن الجينات المقاومة للمضادات الحيوية تحتوى على عدد 23 (701 bp)عند $AphA_{1-3}$ 44.23 ٪ التي تدل على المقاومة لجين الإريثروميسين في الجين نتيجة الجينات المقاومة للامبيسلين هى 12بنسبة (23.07٪) ظهرت عند ***bla*****OXA-61 gene,** (372 bp) .

وأخيرا، يمكن أن نستنتج أن وجود الجينات المقاومة للمضادات الحيوية لميكروبات الكامبيلوباكتر سوف يقلل من فعالية العديد من مضادات الميكروبات المستخدمة في العلاج مما يؤدى إلى ارتفاع معدلات الاصابة المرضية والوفيات المرتبطة بالخسائر الاقتصادية اللاحقة بسبب استخدام مضادات الميكروبات الغير فعالة.