ROLE OF PLASMIDS IN ANTIBIOTIC RESISTANCE OF CAMPYLOBACTER SPP. ISOLATED FROM CHILDREN

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SUMMARY: Six clinical isolates of Campylobacter were examined for the occurrence of conjugative plasmids carrying antibiotic resistance markers. Kanamycin resistance was conjugally transferred from donors to recipients in all cases which indicated that it is plasmid mediated while the resistance to Erythromycin was considered to be chromosomally mediated because it was never transferred by conjugation. The Tetracycline resistance in one Campylobacter strain was encoded on a self-transmissible plasmid, while in other donor strains it was considered to be chromosomally mediated or carried on non-conjugative plasmid because it failed to transfer by conjugation. The Ampicillin and Kanamycin resistance markers of C. jejuni D101 were co-transferred by conjugation to C. coli A9 which indicated that both markers are on the same plasmid.

Occurrence of Ampicillin resistance gene on a plasmid in Campylobacter is reported here for the first time. The Erythromycin and Tetracycline resistance genes of C. hyointestinalis A14 and C. jejuni D101 proved to be chromosomally mediated because chromosomal DNA from both strains were successfully transformed to sensitive strains of Campylobacter which then became resistant to Erythromycin and Tetracycline.

Key Words: Campylobacter, Antibiotic resistance, Plasmid, Conjugation, Transformation.

INTRODUCTION

Members of the *Campylobacter* genus are gram-negative, curved or S-shaped microaerophilic bacteria. They are major pathogenes responsible for human gastroenteritis throughout the world, and enteritis caused by them may exceed, in some places, that of the better known enteric pathogenes such as *Salmonella* and *Shigella* (3,7,11,14). Enteritis due to *Campylobacter* spp, is usually a mild to moderate self-limited diarrheal disease, however patients with severe, prolonged or relapsing *Campylobacter* enteritis are recommended for treatment with antibiotics such as Erythromycin, Gentamycin or Tetracycline, where the Erythromycin is the drug of choice (17,26,28).

An increasing number of antibiotic-resistance *Campylobacter* strains have been identified throughout the world (4,5,25). Studies of antimicrobial resistance in *Campylobacter* spp. range from surveys of resistance patterns of clinical isolates to studies characterizing the genetic determinants that encode the

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resistance phenotypes. *Campylobacter* spp. can harbor a large array of resistance determinants, including several genes that are plasmid mediated (16-19,21,24,28).

Unfortunately clinical laboratories in Iraq do not routinely perform the isolation procedures and antimicrobial susceptibility testing on these important pathogenes. Reports on *Campylobacter* human enteritis in Iraq are very limited (1,13). Moreover, nothing is known about the genetic nature of the antibiotic resistance in local *Campylobacter* strains.

In the present study, we investigated the genetic nature of antibiotic resistance in *Campylobacter* strains isolated in Baghdad to determine whether the different antibiotic resistance markers are located on conjugative plasmids or on the chromosome.

MATERIALS AND METHODS

Bacterial strains

Campylobacter strains used in this study and their resistance pattern are shown in Table 1. There were strains isolated from children with gastroenteritis at Saddam's Central Hospital for Children in Baghdad, and identified by standard biochemical test in a previous study (1).

Media and growth conditions

The following media were used to grow *Campylobacter* strains: Blood agar which contain Columbia blood agar (Oxoid) and 5% blood; Mueller-Hinton (Oxoid); and Brain-Heart Infusion (Oxoid).

Inoculated media were incubated at 37°C in microaerophilic conditions using anaerobic jars.

Antibiotics

The antibiotics used to select, for transconjugants and transformants were Erythromycin (Ery), Kana-mycin (Km), Ampicillin (Ap), Tetracycline (Tc) and Rifadin (Rd) (Winlab limited, UK), and Cephalothin (Cf) (Difco).

Bacterial conjugation

Conjugation between donor and recipient *Campylobacter* strains was performed millipore filters (C. 45 μm) layed on solid media as described by Kotarski *et al.* (6). Transconjugants were selected by plating them on Blood agar containing the appropriate antibiotics (20 μg/ml of each antibiotic). Controls of either donor and recipient cells were run to check spontaneous mutation frequencies.

Isolation of chromosomal DNA

Chromosomal DNA was isolated from the Ery^r, Tc^r strains C. *hyointestinalis* A14 and C. *jejuni* D101 using the quick method of Lema *et al.* (18).

Agarose gel electrophoresis

Samples of DNA in TE buffer were loaded on 0.7% agarose, running conditions were as described in Maniatis *et al.* (9) using TBE buffer. DNA bands were detected by examining the gel under UV light (340 nm).

Table 1: *Campylobacter* strains sed in this study.

Strain	Antibiotic sensitivity pattern	Donor / Recipient	Reference
C. hyointestinalis A11	Km^r, Ery^r, Cf^s	Donor	Ali, 1996
C. hyointestinalis A12	Ery^r, Tc^r, Cf^s	Donor	=
C. hyointestinalis A14	Km^r, Ery^r, Tc^r, Cf^s	Donor	=
C. jejuni D101	Ap^r, Km^r, Tc^r, Ery^r, Cf^s	Donor	=
C. jejuni A13	Km^r, Ery^r, Tc^r, Rd^s	Donor	=
C. coli A15	Ery^r, Rd^s	Donor	=
C. lari A3	Cf^r, Km^s, Ery^s, Tc^s, Ap^s	Recipient	=
C. coli A9	Cf^r, Km^s, Ery^s, Tc^s, Ap^s	Recipient	=
C. jejuni D65	Rd^r, Ery^s, Tc^s, Km^s	Recipient	=

r = resistant

s = sensitive

Transformation

Transformation of *Campylobacter* was performed on Mueller-Hinton agar surface as described by Wang and Taylor (27) with slight modification.

0.2 ml of overnight culture of recipient cells grown in Mueller-Hinton broth were spread on Mueller-Hinton agar and incubated at 37°C for 6 hours under microaerophilic conditions. Aliquotes of donor chromosomal DNA (1 μg DNA in 5 μl TE buffer) were spotted directly onto the inoculated agar without additional mixing or spreading, and incubation was continued overnight. The cells within the DNA spot were scraped up, diluted with Mueller-Hinton broth and spread on Mueller-Hinton agar containing 20 μg/ml of Ery or Tc to select transformants and calculate frequencies. Controls were run in the same manner to score the spontaneous mutations.

RESULTS AND DISCUSSION

Conjugation

Results of the conjugation experiments between different strains of *Campylobacter* are shown in Tables 2 and 3.

Km^r trait was transferred from donors to recipients in all cases, which proved that this marker is plasmid mediated. This result is in agreement with what is known about Km resistance in *Campylobacter* which is usually plasmid mediated (19,24), and occasionally chromosomally mediated (6,10). Molecular studies have identified three different Km^r genes in *Campylobacter* spp., aph A-3 gene located on 48 kb plasmid

Table 2: Conjugation between donor strains C. *hyointestinalis* A11, A12, A14, C. jejuni D101 and recipient strains *C. lari* A3 and *C. coli* A9.

Donor and its resistance pattern	Recipient and its resistance pattern	Antibiotic in selective medium	Conjugation frequency	Donor marker selected
C. hyointestinalis A11 (Km^r, Ery^r)	C. lari A3 (Cf^r)	Km+Cf Ery+Cf	$2.8x10^{-2}$ -	Km^r -
C. hyointestinalis A12 (Tc^r, Ery^r)	=	Tc+Cf Ery+Cf	$5x10^{-2}$ -	Tc^r -
C. hyointestinalis A14 (Km^r, Tc^r, Ery^r)	=	Km+Cf Tc+Cf Ery+Cf	$1x10^{-2}$ - -	Km^r - -
C. jejuni D101 (Ap^r, Km^r, Tc^r, Ery^r)	=	Ap+Cf Km+Cf Tc+Cf Ery+Cf	$3.2x10^{-2}$ $1.1x10^{-2}$ - -	Ap^r Km^r - -
C. hyointestinalis A11 (Km^r, Ery^r)	C. coli A9 (Cf^r)	Km+Cf Ery+Cf	$4x10^{-1}$ -	Km^r -
C. hyointestinalis A12 (Tc^r, Ery^r)	=	Tc+Cf Ery+Cf	$5x10^{-1}$ -	Tc^r -
C. hyointestinalis A14 (Km^r, Tc^r, Ery^r)	=	Km+Cf Tc+Cf Ery+Cf	$1.1x10^{-2}$ - -	Km^r - -
C. jejuni D101 (Ap^r, Km^r, Tc^r, Ery^r)	=	Ap+Cf Km+Cf Tc+Cf Ery+Cf	$2x10^{-2}$ $2.5x10^{-2}$ - -	Ap^r Km^r - -

Table 3: Conjugation between donor strains *C. coli* A15, *C. hyointestinalis* A11, *C. jejuni* A13 and the recipient *C. jejuni* D65.

Donor and its resistance pattern	Recipient and its resistance pattern	Antibiotic in selective medium	Conjugation frequency	Donor marker selected
C. coli A15 (Ery^r)	C. jejuni D65 (Rd^r)	Ery+Rd	-	-
C. hyointestinalis A11 (Ery^r, Km^r)	=	Ery+Rd Km+Rd	- $1.7x10^{-2}$	- Km^r
C. jejuni A13 (Ery^r, Km^r, Tc^r)	=	Ery+Rd Km+Rd Tc+Rd	- $1.2x10^{-2}$ -	- Km^r -

(22), aph-7 located on 14 kb plasmid (23) and aph A-1 which is chromosomally located gene (10). Three of the *Campylobacter* strains used in this study were Km^r, Tc^r, but the two markers never co-transferred to the recipient in any experiment, which indicated that these markers are not located on the same plasmid in any strain. Several studies reported the presence of Km^r gene on plasmid that also encode Tc resistance (6,12).

In contrast to Km^r, the Ery^r marker did not transfer to the recipients in all experiments (Tables 2 and 3). It was concluded that the Ery^r gene is not located on plasmid. This result was expected because, almost, all previous studies have shown that the Ery resistance is unrelated to the presence of plasmid DNA and considered to be a chromosomal gene (18,28). The only report on Ery resistance being a plasmid mediated and conjugally transferrable came from Malaysia (2).

The association of Ery^r gene with the chromosome and not with the plasmid is very important from epidemiological point of view. Erythromycin is the drug of choice for the treatment of *Campylobacter* enteritis, and its unrelatedness to plasmid will decrease the chances of the spreading of the resistance to this antibiotic.

The Tc^r marker of *C. hyointestinalis* A12 was conjugally transferred to *C. lari* A3 and *C. coli* A9 in high frequencies (Table 2). This means the Tc^r gene in strain A12 is carried on a self-transmissible plasmid. The Tc resistance in other donor strains (A13, A14 and D101) did not transfer to the recipients (Tables 2 and 3) which suggested that the Tc resistance in these strains is either chromosomally mediated or carried on a non-selftransmissible plasmid. It is known that the Tc^r determinant in *Campylobacter* spp. is frequently mediated by self-transmissible plasmids of about 45-50 kb in size (15,18). However the Tc^r gene can also be carried on small non-conjugative plasmid (21) or on the chromosome (19).

As a result of conjugation between *C. jejuni* D101 and *C. coli* A9 two types of transconjugants were obtained in similar frequencies, Km^r and Ap^r transconjugants (Table 2). This result indicated that both Km^r

Table 4: Transformation of *C. coli* A9 and *C. jejuni* D65 with chromosomal DNA isolated from *C. hyointestinalis* A14 and *C. jejuni* D101.

Donor and its resistance pattern	Recipient strain	Transformation frequency for Ery^r	Transformation frequency for Tc^r
C. hyointestinalis A14 (Tc^r, Ery^r)	C. coli A9 C. jejuni D65	$4x10^{-1}$ $1.3x10^{-4}$	$2.5x10^{-1}$ $1.1x10^{-4}$
C. jejuni D101 (Tc^r, Ery^r)	C. coli A9 C. jejuni D65	$1.4x10^{-3}$ $4.5x10^{-4}$	$1.2x10^{-3}$ $3.2x10^{-4}$

and Ap^r genes are carried on plasmid in strain D101. We report here the first observation on occurrence of Ap^r marker on a conjugative plasmid in *Campylobacter*. All previous studies reported that the Ap resistance in *Campylobacter* is chromosomally mediated and no plasmid was related to it (19,20).

C. jejuni D101 was the only strain in this study contained two conjugally transferrable antibiotic resistance markers (the Km^r and Ap^r). Whether these markers are located on the same plasmid or not should be clarified. To do this, 50 Km^r transconjugants were streaked on a medium containing Ampicillin. All Km^r transconjugants were also Ap^r, which proved that both markers are located on the same plasmid. Occurrence of Ap^r gene with another antibiotic resistance gene on the same plasmid has never been reported before. Taylor *et al.* (20) found that Ap^r did not co-transfer with Tc^r in *Campylobacter* strain resistant to both Ap and Tc, and therefore they concluded that the Ap^r is chromosomally mediated.

The plasmid of *C. jejuni* D101 carrying Ap^r and Tc^r genes deserves more detailed study to characterize it and determine the nature of the Ap^r gene.

Isolation of chromosomal DNA

The quick method used to prepare chromosomal DNA from *C. jejuni* D101 and C hyointestinalis A14 (8) proved to be efficient and suitable for such purpose. After loading DNA samples on an agarose gel, a single band representing the chromosomal DNA was detected and there were no plasmid bands (data not shown). The DNA concentration was about 0.1 μg/μl and proved to be suitable for transformation experiments.

Transformation

The Ery and Tc resistance did not transfer from *C. hyointestinalis* A14 and *C. jejuni* D101 to the recipient strains during conjugation (Table 2). It was concluded that the resistance to both antibiotics are probably chromosomally mediated in both strains. To confirm this, the chromosomal DNA was isolated from above strains and used to transform the sensitive strains *C. coli* A9 and *C. jejuni* D65 and select for Ery^r and Tc^r transformants. Both recipients were transformed to Ery^r and Tc^r at high frequencies (Table 4). The success of natural transformation with chromosomal DNA reconfirms that the Ery and Tc resistance in these strains are chromosomally mediated.

The conjugation and transformation data proove the following: All Km resistance in studied strains are plasmid mediated, and all Ery resistance are chromosomally mediated. Some Tc^r genes are chromosomally encoded, while others are plasmid encoded. Finally, the Ap^r gene of *C. jejuni* D101 is located on a conjugative plasmid along with the Km^r gene, and this observation is reported for the first time.

Plasmids of local *Campylobacter* isolates should be investigated thoroughly. Determination of plasmid profile and identification and characterization of antibiotic resistance plasmids will certainly help to understand the epidemiology of the disease and the nature of antibiotic resistance.

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