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Complete Elimination of Indigenously Isolated *Agrobacterium Tumefaciens* Strains by Quinolone and Metallo β-Lactam Antibiotics.

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ABSTRACT

The present study aimed to isolate *Agrobacterium tumefaciens* strains from *lycoperiscum esculantum* (Tomato) galls and investigate in vitro the potential killing activities of various antibiotics. Seven *Agrobacterium tumefaciens* strains namely WF2, WF7, WF7d, WF11, WF12, WF14 and WF15 were isolated from galls of different *lycoperiscum esculantum* from various fields in district D.I.Khan. The isolated strains were assayed for their ability to initiate tumors by using a carrot and potato disc assay. Primarly six antibiotics were screened for susceptibility against *Agrobacterium tumefaciens* by a disc diffusion assay. The antibiotic with the widest zones of inhibition were used for determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC).The MIC and MBC of meropenem against almost all strains were the same (0.025mg/ml) except for WF14, (MIC/MBC 0.05mg/ml). The ciprofloxacin was reported as the second most active antibiotic against *A. tumefaciens*. Suppression of the growth of all strains occurred at 0.0031mg/ml except for WF7 and WF14 with MIC 0.0062mg/ml. The MBCs of all strains are the same as the corresponding MICs. However, the strain WF15 was two times higher (0.0093mg/ml). The amoxicillin also represented comparatively lower MIC/MBC (0.2mg/ml) except WF14 (MIC 0.4mg/ml, MBC 0.8mg.ml) without clavulanic acid. It is concluded that both meropenem and ciprofloxacin were highly effective in eliminating the *Agrobacterium tumefaciens* in a low concentration. These findings prove the effective use of ciprofloxacin and meropenem in agrobacterium mediated transformation in plants.

Keywords: *A.tumefaciens*, crown galls, transformation, T-DNA, minimum inhibitory concentration..

INTRODUCTION

Agrobacterium tumefaciens is an aerobic soil-borne, Gram-negative phytopathogen [1,2] and is considered the causative agent for neoplastic outgrowths commonly referred to as crown galls in a number of plants. [3] It overwinters in infested soils and remains saprophyte for several years. [4]

The *Agrobacterium tumefaciens* is being successfully utilized as an effective tool for plant genetic engineering on account of its ability to bear the transfer and integrate a T-DNA host (a dicot plant) genome. Such transformation, suppression and elimination of agrobacteria in host tissues is not only mandatory to avoid release of genetically modified microorganisms but also necessary to lessen the harmful effects on the growth and regeneration of plant tissues. [5, 6] There are no chemical pesticides available against *A. tumefaciens,* except biological control that can provide protection to some extent. The biological approach is accompanied by the immersion of the roots and crown in a solution containing genetically modified bacteria like *Agrobacterium radiobacter* strain 84 before planting. [7]

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Likewise β-lactam antibiotics including carbenicillin and cefotaxime are commonly used to eliminate the *A.tumefaciens* from plant tissues. The β-lactams bind to penicillin- binding proteins (PBPs) [8] and interfere with the biosynthesis of the peptidoglycan component of the bacterial cell wall [9] thus resulting in cell lysis. [10] However, such antibiotics are not only expensive, but are also needed in high concentrations either alone or in combination (carbenicillin at 500 mg/l, cefatoximes at 200–300 mg/l).[11]

The search for an effective antibiotic with the aim to eliminate *Agrobacterium tumefaciens* is an old pursuit, [12, 13] and numerous antibiotics like ticarcilline, carbenicillin, cefatoxime, erythromycin, spectinomycin, polymyxin B, chloramphenicol, methicillin, augmentin 500, augmentin 250, and moxalactam are being assayed for their activity, but the question whether or not *Agrobacterium* has been completely eliminated still remains unanswered. Yet, in some cases the use of a combination of antibiotics is recommended. [14]

Therefore, there is a need to screen some alternative antibiotics that are highly effective in the complete elimination of *A.tumefaciens*. This present study was aimed to isolate *A. tumefaciens* from *lycopersicum esculentum*, detect the tumor initiating *A. tumefaciens* strains and screen a suitable antibiotic that completely eliminates these strains.

Results and Discussions

The susceptibility of selected antibiotics was determined using a disc diffusion method as summarized in table (1), which ranged from 0 to 14mm.The meropenem (12-14mm) represented the widest zones of inhibitions against all strains of *A. tumefaciens* followed by ciprofloxacin (11-13mm) with only a narrow difference in the zones of inhibition. The streptomycin (4-5mm), ceftrioxone (5-7mm) and amoxicillin (4-6mm) has shown almost equal sensitivity to *A. tumefaciens* but comparatively small zones of inhibitions than both meropenem and ciprofloxacin. The azteronam (0mm) and cefepime (0-2mm) were observed to have no or very little susceptibility towards *A. tumefaciens.*

Table: 1 Zones of inhibition of *Agrobacterium tumefaciens* against antibiotics.

Antibiotics	Zones of inhibition(*mm*)*						
	WF2	WF7	WF7d	WF11	WF12	WF14	WF15
ciprofloxacin	12	13	12	13	11	13	13
ceftriaxone	7	5	6	5	6	5	7
cefepime	2	2	2	1	2	1	0
meropenem	14	14	13	14	13	12	13
amoxicillin	6	5	4	5	4	5	6
aztreonam	0	0	0	0	0	0	0
streptomycin	4	4	5	4	4	5	4

* *mm* millimeter

Based on the susceptibility results from a disc diffusion assay, only four antibiotics, namely meropenem, ciprofloxacine, amoxicilline and ceftriaxone, were selected for determination of MIC and MBC which is presented in table (1) and (2), respectively. The β-lactam antibiotics are best known for their activity against *A. tumefaciens.* [15] The most commonly used antibiotics in *A. tumefaciens* mediated transformation experiments are the carbenicillin and cefotaxime (β-lactam antibiotics). [8] However, there exists multiple problems like β-lactam ring hydrolysis by β-lactamase enzymes produced by the microorganisms which limit their use. Thus, in the search for the best β-lactam antibiotic, ceftriaxone was screened against all *A. tumefaciens* strains. It was surprisingly observed that the results of MIC and MBC were significantly greater (>1.6 mg/ml) than compared to earlier reports for other β-lactams antibiotics [10] which were mostly the result of β-lacatmases production by gram negative pathogens. [16] On the other hand, the amoxicillin represented much better susceptibility against *A. tumefaciens* (MIC 0.2mg/ml and same corresponding MBC) except WF14 (MIC 0.4mg/ml, MBC 0.8mg.ml) which was comparatively higher than described previously [17,18] when amoxicillin was used in combination with clavulanic acid (β-lacatmases inhibitor). Likewise, the present study confirms susceptibility of amoxicillin against *A. tumefaciens*.

Table 2 minimum inhibitory concentration (MIC) of selected antibiotics against *Agrobacterium tumefaciens.*

Antibiotics	MIC (mg/ml)*						
	WF2	WF7	WF7d	WF11	WF12	WF14	WF15
ciprofloxacin	0.0031	0.0062	0.0031	0.0031	0.0031	0.0062	0.0031
ceftriaxone	0.8	1.6	1.6	1.6	1.6	1.6	1.6
meropenem	0.025	0.025	0.025	0.025	0.025	0.05	0.025
amoxicillin	0.2	0.2	0.2	0.2	0.2	0.4	0.2

*MIC minimum inhibitory concentration, mg/ml milligram/milliliter

Table: 3 Minimum bactericidal concentrations (MBC) of antibiotics against *agrobacterium tumefaciens*

Antibiotics	MBC (mg/ml)*						
	WF2	WF7	WF7d	WF11	WF12	WF14	WF15
ciprofloxacin	0.0031	0.0062	0.0031	0.0031	0.0031	0.0062	0.0093
ceftriaxone	1.6	>1.6	>1.6	>1.6	>1.6	>1.6	>1.6
meropenem	0.025	0.025	0.025	0.025	0.025	0.05	0.025
amoxicillin	1.6	>1.6	>1.6	>1.6	>1.6	0.8	>1.6

*MBC minimum bactericidal concentration , mg/ml milligram per milliliter

The meropenem is an effective antibiotic and its efficacy against *Agrobacterium tumefaciens* mediated transformation has been established previously[7] despite the presence of a β-lactam ring. In the present study, meropenem showed the highest activity (0.025mg/ml) in suppressing all tested *A. tumefaciens* strains except one (WF14, MIC 0.05mg/ml which is same as MBC).The corresponding MBC for all other strains are of equal concentration (0.025mg/ml), which strongly supports the previous reports. [19] However, the present study reveals significantly lower MIC and MBC values than earlier reports. [20] The geographical location, nature of plant variety, production of carbapenamases by strains and adaptations of tested strain could be contributing factors in this reference. This strong antimicrobial activity of meropenem is due to the presence of a zwitterionic (presence of positive electrical charges) structure which mainly resists its degradative hydrolysis. [21]

The quinolone are best known for their gram negative microorganism eliminating activities like *Agrobacterium tumefaciens.* [22] In the present study, ciprofloxacin (quinolone) was proven as the second most highly active antibiotic against tested *A.tumefaciens* strains. Suppression of growth of almost all strains occurred at 0.0031mg/ml except WF7 and WF14 with MIC 0.0062mg/ml. The MBC of all strains are the same as the corresponding MIC. However, the strain WF15 had two times higher (0.0093mg/ml) MBC than MIC.[23] An extensive literature review reveals no data regarding MIC and MBC of ciprofloxacin against *A.tumefaciens* isolated from gall tissues of dicot plants. However, the MIC and MBC values of ciprofloxacin determined in the present study were the same as previously reported in patients suffering from *A. tumefaciens* infections.[24] This study highlights ciprofloxacin as the second most active antibiotic in the elimination of *Agrobacterium tumefaciens* from gall tissues.

General Experiment

The crown gall tissues were separated from tomato plants (*Lycopersicum esculentum*) cultivated in the fields of district D. I. Khan. The samples were aseptically transferred in sterilized containers to the laboratory.

Crown gall extract preparation

The crown gall tissue samples (12gm each approximately) were rinsed in DDW (doubled distilled water) for the removal of soil and toxic materials. Afterwards, the galls were sterilized with household bleach (clorox 10%, Beecham Pakistan (pvt) Ltd) for 2-5 minutes, washed 3 to 5 times with DDW, chopped and immersed in DDW overnight at 28-30°C.

After overnight incubation at 28-30°C, the extracts were inoculated onto selective media including MacConkey agar (Oxoid), [25] Clark's selective media (Oxoid), and MGY agar medium (Sigma). [26] Colonies were examined for color development, growth and fermentation**.** The isolated colonies were maintained on Luria-Bertani (LB) agar medium (Oxoid) for further experiments.

Agrobacterium Strains:

Seven *A. tumefaciens* strains namely WF2, WF7, WF7d, WF11, WF12, WF14a and WF15 were isolated from tomato crown gall and identified according to procedures developed by Bergeys' manual of determinative bacteriology, 9th edition. Standard *A. tumefaciens* was obtained from the Department of Biochemistry, Quaid I Azam University, Islamabad, Pakistan.

Tumor development assay

a) Carrot bioassay

A tumor development assay was performed using freshly collected carrots (*Daucas carota L.*) from a local market in D. I. Khan city (KPK) Pakistan. The carrots were first surface sterilized with commercial bleach (clorox 10%, Beecham (pvt) Ltd, Pakistan) followed by washing three times with DDW water. Carrot discs were prepared (5×8 mm) aseptically using a sterilized cutter and placed on the nutrient agar containing petri plates (15g/litre) for bioassay. Each carrot disc was then supplied with 100ul of appropriate *A. tumefaciens* inoculums (10^8 cfu/ ml). The petri plates containing the carrot discs loaded with A. Tumefaciens inoculum were sealed with parafilm and incubated at 27-30°C. After 3 weeks, discs were checked for young gall (tumor) development from meritmatic tissues around the central vascular system. [27]

b) Potato Disc Bioassay

Red skin potatoes (*Solanum tuberosum L., solanaceae*) were purchased from a local market in D. I. Khan city, (KPK) Pakistan. Potatoes were first surface sterilized (clorox 10%, Beecham (pvt) Ltd, Pakistan). Potato discs were prepared (5×8 mm) aseptically and placed on the freshly prepared nutrient agar (Oxiod) plates (15g/litre). Each disc was supplied with 50µl of *A. tumefaciens* inoculums (10^8 cfu/ml). The petri plates were sealed by parafilm and incubated at room temperature

(25-30°C) for 3 weeks. After the incubation period, the discs were stained with lugol's iodine solution (10%KI and 5% I_2) for 30 minutes and tumors were observed under a sterio microscope where the tumor cells lack starch. [28]

Antibiotics

Seven different antibiotic discs were used namely, ciprofloxacin (Oxoid), ceftriaxone (Oxoid), meropenem (Oxoid), cefepime (Oxoid), streptomycin (Oxoid), amoxicillin (Oxoid) and aztreonam (Oxoid). For MIC and MBC studies, the required active pharmaceutical ingredients (API) were obtained commercially; ciprofloxacin (Bayer (pvt) Ltd, Pakistan), ceftrioxone (Sanofi Aventis (pvt) Ltd, Pakistan), meropenem (Astra Zeneca. UK), and amoxicillin (Glaxo, Smithcline and Beecham (pvt) Ltd, Pakistan).

Determinations of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of the antibiotics were determined by the agar dilution method. [29, 30] The sterilized LB agar (Oxoid) was prepared and allowed to cool to 50°C. After cooling at 50°C about 19 ml of this media was added to sterilized test tubes each containing 1ml of a different concentration of antibiotics. The mixture was thoroughly mixed and poured into pre-labeled sterile petri dishes. The petri plates having only growth media were prepared in the same way to serve as a control. The concentrations of the antibiotics used in this test ranged from 1.6 mg to 0.01 mg/ml. Suspensions of the *Agrobacterium tumefaciens* having density adjusted to 0.5 McFarland turbidity standard (10^8 cfu/ml) were inoculated onto series of L.B agar plates. The plates were then incubated at 25-30°C for 72 to 96 hours. The lowest concentration which inhibited the growth of the respective organisms was taken as MIC. All tests were carried out in triplicate.

Minimum Bactericidal Concentration (MBC)

MBC was defined as the lowest drug concentration producing a survival rate of <0.1%. The MBC of the selected antibiotics was measured by the viable cell count method [31] and the results were expressed as the number of viable cells as a percentage of the control.

Conclusion

The present study concludes that the meropenem and ciprofloxacin are best suited for the complete elimination of *Agrobacterium tumefaciens.* The study further proposes these antibiotics for use in *Agrobacterium tumefaciens* mediated plant transformation experiments.

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التلخص الكلي من بكتيريا اقروبكتيرم كومي فاسنر بواسطة مضادات الكويتولوتز والبيتالكتامن

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ملخـص

تهدف الدراسة الحالية إلى عزل بكتيريا اقروبكتيرم من الطماطم ودراسة فاعلية مجموعة مضادات حيوية عليها. تم عزل 7 سلالات من الأقربكتيرم وهي WF2, WF7, WF7D,WF11,WF12,WF14 و WF15 من مجموعات مختلفة من الطماطم مزروعة بحقول مختلفة بمقاطعة D.IKhan.

تم دراسة قدرة هذه السلالات على إحداث أورام باستعمال قرص الجزر والبطاطا. تم تحديد التركيز القاتل الأقل للمضادات التي لها اوسع مجال قاتل للبكتيريا. اظهر مضاد الميروبنيم أعلى فعالية ممكنة حيث كان له التركيز القاتل الأقل يساوي 0.025 ملغم لكل من مل ضد جميع السلالات ما عدا سلالة WF14 حيث كان التركيز القاتل الأقل يساوي 0.05 ملغم لكل مل. المضاد الحيوي سايروفلوكاسين حل ثانياً في فاعليته ضد اقروبكتيرم. المضاد الحيوي امكسلسين كان الأضعف ضد اقروبكتيرم. مما سبق يمكن استنتاج أن كل من الميرديرم والسايبروفلوكاسين قد اظهرا فاعلية عالية ضد الاقرويكزم وبالتالي تم استعمالها لمكافحة هذه البكتيريا في النباتات.

الكلمات الدالة: السايروفلوكاسين، الأقروبكتيرم، التركيز القاتل الأقل.

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