ANTIBACTERIAL ACTIVITIES OF MULTI DRUG RESISTANT MYROIDES ODORATIMIMUS BACTERIA ISOLATED FROM ADULT FLESH FLIES (DIPTERA: SARCOPHAGIDAE) ARE INDEPENDENT OF METALLO BETA-LACTAMASE GENE

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ABSTRACT

Flesh flies (Diptera: Sarcophagidae) are well known cause of myiasis and their gut bacteria have never been studied for antimicrobial activity against bacteria. Antimicrobial studies of *Myroides* spp. are restricted to nosocomial strains. A Gram-negative bacterium, Myroides sp., was isolated from the gut of adult flesh flies (Sarcophaga sp.) and submitted to evaluation of nutritional parameters using Biolog GN, 16S rRNA gene sequencing, susceptibility to various antimicrobials by disc diffusion method and detection of metallo βlactamase genes (TUS/MUS). The antagonistic effects were tested on Gram-negative and Gram-positive bacteria isolated from human clinical specimens, environmental samples and insect mid gut. Bacterial species included were Aeromonas hydrophila, A. culicicola, Morganella morganii subsp. sibonii, Ochrobactrum anthropi, Weissella confusa, Escherichia coli, Ochrobactrum sp., Serratia sp., Kestersia sp., Ignatzschineria sp., Bacillus sp. The Myroides sp. strain was resistant to penicillin-G, erythromycin, streptomycin, amikacin, kanamycin, gentamycin, ampicillin, trimethoprim and tobramycin. These strain showed antibacterial action against all bacterial strains except W. confusa, Ignatzschineria sp., A. hydrophila and M. morganii subsp. sibonii. The multidrug resistance of the strain was similar to the resistance of clinical isolates, inhibiting growth of bacteria from clinical, environmental and insect gut samples. The metallo β-lactamase (TUS/MUS) genes were absent, and resistance due to these genes was ruled out, indicating involvement of other secretion machinery.

Key-words: Flesh fly, *Myroides* sp., 16S rRNA, antibacterial activity, metallo beta-lactamase

INTRODUCTION

The genus *Flavobacterium* was created in 1923 and since then, the taxonomy of this genus has undergone substantial changes, especially for strains belonging to the former *Flavobacterium odoratum* (*F. odoratum*) species. The *F. odoratum* was further classified afterwards in a separate genus on the basis of its unique phenotypic features. Strains of this species, differing from most *Flavobacterium* species in being nonsaccharolytic, fail to produce indole (10). Using 16S rRNA

sequencing *F. odoratum* was shown to occupy an independent taxonomic position in the genus *Flavobacterium* (34). Finally, the genus *Myroides* was created for organisms removed from the genus *Flavobacterium* on the basis of hybridization experiments and phenotypic characteristics and two species have been delineated; *Myroides odoratus* (*M. odoratus*) and *Myroides odoratimimus* (*M. odoratimimus*) (34). Antimicrobial properties of Flavobacteria are well studied (5,12) up to molecular and kinetic level (18). Bacteria *Myroides* spp. are aerobic, yellow-pigmented, Gram-negative rods that grow at both room

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temperature and 37°C. They are habitat-specific organisms, like other members of the Flavobacteriaceae family, and are found in wet environments (12), insect guts (31) and sea water (19,37). Strains of M. odoratus and M. odoratimimus are sources of nosocomial infections in humans and behave like low-grade opportunistic pathogens. Myroides was identified as a cause of surgery wound and urinary tract infections, septicemia, pneumonia, meningitis, fasciitis, and ventriculitis (36). Although the antibiotic resistance patterns of Flavibacterium and Myroides strains shows variable susceptibility to beta-lactams (β-lactams) (12), there is constant decrease in susceptibility to cephalosporin and imipenem. The β-lactamases produced by Gram negative and Gram positive bacteria play a significant role in resistance against \(\beta \)-lactam antibiotics but with variable activities (14,18). Previously, M. odoratus was isolated from lepidopteran gut and it was hypothesized that this bacteria may be involved in the production of some biosurfactants (31). However, there is not a single report of isolation as well as antimicrobial studies on M. odoratimimus of insect gut origin, since all previous reports are from clinical samples. It is well known fact that Sarcophagids secrete antibacterial proteins, inhibiting entrance of pathogens in their gut contents (1). Moreover these flies have also been linked with myiasis like infections (3).

During the course of insect gut microbiota studies, we isolated a *Myroides* sp. from adult flesh flies (Diptera: Sarcophagidae) collected from chicken bait and characterized by various genotypic and biochemical methods. The antimicrobial susceptibility testing and antimicrobial activity of this bacterium has been discussed. Bacterial isolates belonging to different lineages (data not shown) were also isolated from larval stages of these flies. Since *Myroides* sp. was exclusively documented in adults and in flies' guts and previously documented for antimicrobial action, here we present the data from adults only.

MATERIALS AND METHODS

Isolation of bacteria from flesh flies guts

The larval flesh flies were reared on chicken flesh kept in wire mesh cages to obtain adult flies and a few adult flies were collected directly from the flesh market in Pune, India, using insect net and brought alive to the laboratory in PET jars during June 2007. To achieve this, partly cooked fresh chicken flesh was kept in open petridish in the window of the laboratory to attract the flies. After the visit of a few sarcophagid flies, the flesh was transferred to wire mesh cages or transparent PET bottles covered with wire mesh and the larvae were allowed to grow to maturity and pupate at room temperature. The flies that hatched were collected and used in the studies. All this was done in Pune metropolis, Maharashtra State, India. About 10 flies were tested. Gut contents were aseptically excised and

placed in physiological saline. The minced gut suspension was poured on Luria agar (USB) supplemented with 5% defibrinated and inactivated human blood. The plates were incubated aerobically at 28°C and 37°C for 18-24 hours. The bacteria (named as FFA2) with characteristic yellow pigmentation were Gram stained, sub cultured on blood agar plates and stored in 80% glycerol solution at -80°C following the standard protocol mentioned elsewhere. However, we present the data from adult stage in the present investigation.

PCR amplification, sequencing and phylogenetic analysis

Genomic DNA from the broth culture was prepared according to Sambrook et al., 1993 (28). The 1500 bp fragment of 16S rRNA gene was amplified from extracted DNA using bacterial universal primers specific to 16S rRNA gene, primer 27F, 5'-CCA GAG TTT GAT CMT GGC TCA G-3' (binding at 5th base of Escherichia coli numbering system of Brosius et al. 1978 (4)) and primer 1525R, 5'-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC -3' (binding at 1495th position of E. coli). PCR and DNA sequencing protocols have been described earlier (23). The 16S rRNA gene sequence of strain FFA2 was compared with the sequences obtained from GenBank. Sequences were aligned with Clustal W software (32) and evolutionary distances along with Knuc value (16) were generated. Alignment gaps and ambiguous bases were not considered while comparing 1349 nucleotides of 16S rRNA gene. A phylogenetic tree was constructed by using neighbourjoining method (27) and Kimura 2 parameter in MEGA v3.1 (16). The topology of the phylogenetic tree was evaluated by the bootstrap re-sampling method of Felsenstein (8) with 1000 replicates (Fig. 1).

Biochemical identification of isolate FFA2 by BIOLOG-GN

Nutritional parameters of strain FFA2 was determined by using BIOLOG-GN microplates as described in Yoon *et al.*, 2006. The protocols were followed as per manufacturers' instructions.

Antibiotic sensitivity analysis of FFA2

The antimicrobial susceptibility testing was performed using a broth dilution method on Mueller Hinton agar (Hi-Media Laboratories, India) as recommended by National Committee for Clinical Laboratory Standards (NCCLS) (22) with various antimicrobial agents including those described by Holmes *et al.* 1979 (12). In all, 43 antibiotics were tested for antibiotic sensitivity on FFA2.

Antimicrobial activity testing using agar well method and paper disk diffusion method

The antibacterial activity of isolate FFA2 was checked by plating reference isolates (see Table 1 for details) individually on Luria broth and sub-cultured on Mueller Hinton agar (Hi

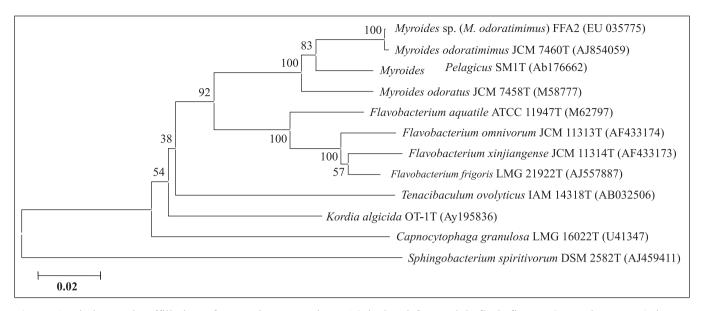


Figure 1. Phylogenetic affiliation of *Myroides* sp. strain FFA2 isolated from adult flesh fly gut *Sarcophaga* sp. (Diptera: Sarcophagidae). Neighbor joining tree constructed in MEGA v3.1 using Kimura 2 parameter using *Myroides* sp. isolate FFA2 and related bacteria based on 16S rRNA gene sequence comparisons. *Sphingobacterim pritovorum* DSM 2050^T was used as an outgroup. Bootstrap replicates of 1000 numericals are shown on nodes. Bar 0.01 indicates nucleotide substitutions per site.

Table 1. Comparative biochemical characteristics of *Myroides* sp. strain FFA2, *M. odoratimimus* JCM 7460*T* (Holmes *et al.*, 1979) and *M. odoratus* JCM 7458*T* (Vancanneyt *et al.*, 1996).

Characteristic	Myroides sp. Isolate FFA2	<i>M. odoratimimus</i> JCM 7460 ^T	<i>M. odoratus</i> JCM 7458 ^T
Source	Sarcophaga sp.	Wound swab	Faeces
Cell morphology	Short rods	Rods	Long rods
Cell diameter (um)	2.5–3	3.5-4	11–12
Cell width (um)	0.2-0.3	0.2-0.3	0.2-0.3
Pigmentation	Yellow	Pale yellow	Yellow
NaCl range	0–7	0–6	0–5
pH range	6–9	6–9	6–9
Catalase	+	+	+
Urease	+	+	+
Nitrate reduction	+	+	+
Activity of:			
Esterase (C4)	+	+	+
Esterase lipase (C8)) +	+	+
Utilization of:			
L-Histidine	+	+	+
α-Hydroxybutyric a	acid –	_	+
Succinamic acid	+	+	+
Urocanic acid	+	+	+

Media Laboratories, India). Macerated adult gut content was also tested for antimicrobial activity. Each reference strain was plated at equal bacterial cell density, determined by taking optical density (OD) at 600 nm in spectrophotometer (BioRad, Hercules, USA) to get mat growth followed by the filter paper disk soaked in FFA2 culture was placed at the centre of each plate. Simultaneously, FFA2 bacterial suspension was also added in the wells cut on agar. All the plates were incubated at 28°C and 37°C for 24 h. Bacteria strains were glycerol preserved at -80°C.

Detection of TUS/MUS genes in Myroides sp.

In order to check presence of β-lactamase gene (1500 bp), which is known to be responsible for variable resistance pattern to β-lactam antibiotics and decreased susceptibility to carbapenems in various Flavobacteria, the PCR was performed by the specific primers spanning entire gene along with open reading frame (ORF) (Mammeri *et al.* 2002). The PCR was carried out in 25 ul volume containing 2.5 mM nucleotides (dNTPs), 10X PCR buffer, 10 pM of forward and reverse primers, 1U of *Taq* DNA polymerase and 10 ng genomic DNA. Cycling parameters were initial denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for

1 min, and the cycles were repeated for 35 times. Amplicons were electrophoresed in 1% agarose gel followed by their visualization under UV light.

RESULTS

16S rRNA gene sequence of strain FFA2 (GenBank accession number EU 035775) showed highest similarities to the sequences of *M. odoratimimus* JCM 7460^T, AJ854059 (100%) and *M. odoratus* JCM 7458^T, M58777 (95%) and *M. pelagicus* IAM 15337^T, AB176662 (96%). The phylogenetic tree showed that the strain FFA2 clustered with *M. odoratimimus* JCM 7460^T with 100% bootstrap support. These results indicated that FFA2 strain is *M. odoratimimus* JCM 7460^T (12) of genus *Myroides*. Biochemical characteristics of strain FFA2 and *M. odoratimimus* JCM 7460^T were compared. Strain FFA2 was able to grow at 7-9% NaCl, whereas previously reported clinical isolates could tolerate 5-6% NaCl concentration and recently reported isolate *M. pelagicus* IAM 15337^T could tolerate 9% NaCl. Moreover, the strain FFA2 showed some biochemical characters similar to *M. odoratimimus* as shown in Table 2.

The strain FFA2 was resistant to antimicrobials like gentamycin (10 mcg), ceftriazone (30 mcg), cefaclor (30 mcg), penicillin G (10 Units), erythromycin (15 mcg), aztreonam (30 mcg), streptomycin (25 mcg), amikacin (30 mcg), co-trimoxazole (25 mcg), kanamycin (30 mcg), ampicillin (10 mcg), ceftazidime

(30 mcg), netillin (30 mcg), ticarciliin (75 mcg), trimethoprim (5 mcg), suplhomethoxazole (25 mcg), cephalothin (30 mcg), cefoxitin (30 mcg), tobramycin (10 mcg), piperacillin (100 mcg), teicoplanin (30 mcg) (Table 3). Whereas, isolate was sensitive to antimicrobials like gatifloxacin (30 mcg), vancomycin (30 mcg), ofloxacin (5 g), sparfloxacin (5 mcg), azithromycin (15 mcg), doxycycline hydrochloride (30 mcg), chloramphenicol (30 mcg), tetracyclin (30 mcg), ciprofloxacin (5 mcg), nitrofurantoin (300 mcg), norfloxacin (10 mcg) by disk diffusion method.

Gram negative Myroides sp. isolated in present case exhibited antibacterial activity against bacteria like O. anthropi LMG 3331^T (clinical isolate), A. culicicola MTCC 3249^T (mosquito gut isolate), E. coli JM109 (Genetically engineered strain), Serratia sp. strain ST11 isolated from beetle gut (Stibara sp.), Kerstersia sp. strain HF2 (isolated from Musca domestica gut), Bacillus sp. strain FFL7 (isolated from flesh fly larvae). Whereas, it could not inhibit the growth of clinical isolates like M. morganii subsp. sibonii CDC 8103-85, A. hydrophila ATCC 7966, Ignatzschineria sp. and O. intermedium (MTCC 4990) strain M86 and Gram positive bacteria like W. confusa ATCC 14434^T. Minced gut content inhibited growth of only two bacterial strains namely, Bacillus sp. isolate FFL7 and Kerstersia sp. strain HF2. Strain FFA2 found to be negative for the presence of metallo β -lactamase (TUS/MUS) gene (1.5 Kb), which is known to be involved in variable resistance to B-lactams and carbapenems.

Table 2. Bacterial strains used in the study along with their source of isolation, type strain, 16S rRNA gene accession numbers available in GenBank.

Bacterial Strains	Type strain	GenBank accession number	Source of isolation	Pathogenic (Yes/Not proved)	Reference
Myroides sp. strain FFA2	Not assigned	EU035775	Flesh fly (Sarcophaga sp.)		
			adult gut	N	This paper
Aeromonas hydrophila					
Hybridization group (HG) 1	ATCC 7966 ^T	X60404	Human clinical specimen	Y	(30)
Aeromonas culicicola subsp. SH	MTCC 3249 ^T	AY130991	Mosquito (A. aegyptii) midgut	N	(23)
Morganella morganii subsp.					
sibonii Biogroup 2	DSM14850 ^T	DQ358146	Human clinical specimen	Y	(15)
Ochrobactrum anthropi	$LMG3331^{T}$	AM114398	Human clinical specimen	Y	(11)
Weissella confusa NCDO 5186	ATCC 14434 ^T	X52567	Grass silage	N	(13)
Ochrobactrum sp. strain M86	MTCC 4990 ^T	DQ100421	Clinical isolate	N	(7)
Escherichia coli strain JM109	Not assigned	AB305017	Genetically engineered	N	Promega
Serratia sp. strain ST11	Not assigned	EF600986	Beetle (Stibara sp.) midgut	N	Unpublished
Kestersia sp. strain HF2	Not assigned	Awaited	House fly (Musca domestica) gut	N	Unpublished
Ignatzschineria sp. strain FFA1	Not assigned	EU008088	Flesh fly (Sarcophaga sp.) adult gu	ut N	Unpublished
Bacillus sp. strain FFL7	Not assigned	Awaited	Flesh fly (Sarcophaga sp.) larval g	ut N	Unpublished

Table 3. The diameters of zone of inhibitions of antibacterial activity (in mm) of FFA2 with other bacterial strains included in the study.

Bacterial Strains tested	Zone of inhibition size (mm) due to <i>Myroides</i> sp. FFA2	Growth inhibited <i>Myroides</i> sp. by (Yes/No/Weakly)	Growth inhibited by Gut content (Yes/No/Weakly)
Aeromonas hydrophila Hybridization group (HG) 1	7	N	N
Aeromonas culicicola subsp. SH	19	Y	Y
Morganella morganii subsp. sibonii Biogroup-2	10	N	N
Ochrobactrum anthropi	20	Y	Y
Weissella confusa	7	N	N
Ochrobactrum sp. strain M86	9	N	N
Serratia sp. strain ST11	19	Y	Y
Kestersia sp. strain HF2	21	Y	Y
Ignatzschineria sp. strain FFA1	8	N	N
Bacillus sp. strain FFL7	19	Y	Y
Escherichia coli strain JM109*	18	Y	Y

^{*} Values were compared with NCCLS recommendations with Escherichia coli as a control.

DISCUSSION

Flavobacterium species (M. odoratus and M. odoratimimus), glucose-non fermenting, Gram-negative rods, are widely occurring in natural ecosystems, like soil, fresh and marine waters, in foods and in sewage treatment plants. As Holmes (12) stated, "The problem of treating infections caused by gentamicin-resistant Flavobacterium spp. remains unsolved" looks valid statement. As a result of the wide variation in antimicrobial susceptibility shown by different species, a test on susceptibility to different antibacterial agents is essential in order to select an adequate therapy. The marked multiple drug resistance observed in some species, prompts the need to develop new antimicrobial agents active against this group of bacteria and to search for synergistic combinations. Resistance of nosocomial originated Flavobacteria to wide range of antimicrobial agents like gentamycin, tobramycin, amikacin and carbenicillin to which Gram-negative, non fermentative bacteria might be expected to be susceptible to suggests that any infections due to these species would prove difficult to treat, in case of clinical cases involving systemic infections (9,12).

The β -lactamases produced by Gram-negative and Gram-positive bacteria play vital role in resistance against β -lactam antibiotics (29). Mammeri *et al.* (18) proposed that although *Myroides* spp. possess metallo β -lactamases, it is difficult to predict the role of β -lactam resistance in pathogenecity and epidemiology. This might be due to the reason that since metalloenzymes expressed in *E. coli* gives much lower level of β -lactam resistance as compared to original producers. Such phenomenon is observed due to combined biosynthesis of carbapenem derivatives and carbapenem hydrolyzing β -lactamases as observed in *Streptomyces* sp. Bacteria isolated

from lepidopteran gut are known to produce biosurfactants and act as plant volatiles; however, their exact role is not yet understood, but it is hypothesized that they might have the same role as in case of mammals (19). Present investigation is the first documented evidence of presence of M. odoratimimus in adult flesh flies (Sarcophagidae) of diptera. Prevoiusly, a Myroides sp. (M.odoratus) was isolated from lepidopteran guts. We for the first time investigated the antimicrobial activities of our *Myroides* sp. in various clinical, environmental and insect gut bacterial isolates. Myroides bacteria isolated from flesh fly guts showed resistance to various antimicrobials including the one which were described in Holmes et al., 1979 and proved to be multi drug resistant. Although, β-lactam resistance has been linked with presence of β -lactamase genes, according to our interpretation, the Myroides sp., in our samples (FFA2) lacked presence of TUS and MUS genes (18). Strain FFA2 isolated from flesh fly gut could not inhibit the growth of Gram positive bacteria like Weissella confusa, which was isolated from food samples (2). The growth of Gram negative bacteria like O. anthropi LMG 3331^T (clinical isolate), A. culicicola MTCC 3249^T (mosquito gut isolate), E. coli JM109 (Genetically engineered strain) was inhibited, even though these bacteria possess β-lactamase and conjugation related secretion mechanisms (11,21,24). Interestingly, FFA2 strain inhibited growth of some of the recently isolated gut bacteria from insects (unpublished data) like Serratia sp. strain ST11 isolated from beetle gut (Stibara sp.), Kerstersia sp. strain HF2 (isolated from Musca domestica gut), Bacillus sp. strain FFL7 (isolated from flesh fly larvae). Bacteria belonging to genera like Serratia, Kerstersia and Bacillus are well documented for their pathogeneticity from various environments (6,17,25). Some of the clinical isolates like M.

morganii subsp. sibonii CDC 8103-85, A. hydrophila ATCC 7966 and O. intermedium (MTCC 4990) strain M86 could not be inhibited by the strain FFA2. The M. morganii, A. hydrophila, Ochrobactrum spp. are well known for β-lactamase gene (21,33,35). Recently isolated strain of *Ignatzschineria* sp. from insect gut (unpublished data) was resistant to the antimicrobial action of Myroides sp. Interestingly, Ignatzschineria sp (formerly Schineria) is a bacterium already known to be associated with human infections (20). Further, extract of gut content also inhibited the growth of Bacillus sp. and Kerstersia sp. indicating the production of antibacterial compounds by this fly in order to survive and combat the infestations due to bacteremia by other bacterial pathogens (1). However, why the *Myroides* sp. isolated in the present investigation did not inhibit growth of some bacteria isolated from same fly, however, it could be the result of interaction between flesh fly and Myroides sp., which is unknown, which might have conferred resistance. We supported this fact that there must be some reason to believe due to association between fly and bacteria, due to which survival is possible in the fly gut irrespective of providing resistance to the host (i.e. fly) but in vitro activity exists outside the gut.

It is noteworthy that *Myroides* sp. isolated in present case, exhibited broad range of antibiotic resistance and so also antimicrobial activity against various pathogens. It is well established fact that the resistance to various antimicrobials may be due to presence of some virulence gene or involvement of secretion machinery of multi drug efflux proteins (12,24). Increased antibiotic resistance in such bacteria where βlactamase gene subunit is lacking, is mediated by acquiring resistance mechanisms through mutations in bacterial genome or by gaining additional genes through horizontal gene transfer or by physiology dependent resistance. Moreover, bacteria have intrinsic resistance mechanisms that are often not detected in standard antibiotic sensitivity tests performed at laboratory level (26). However, Myroides are linked with low virulence and mortality when it comes to clinical level. Here we propose that some antibiotics like gatifloxacin, vancomycin, ofloxacin, sparfloxacin, azithromycin, doxycycline, chloramphenicol, tetracyclin, ciprofloxacin, nitrofurantoin and norfloxacin could be useful while treating the Myroides infections caused by 'visiting' dipteran flies. Since flesh flies are vectors for myiasis like infections, we would like to warn that the consumption of meat infected with multidrug resistant M. odoratimimus carried by flesh flies might lead to severe consequences.

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RESUMO

Atividades antibacterianas de *Myroides odoratimimus* isolada de moscas varejeiras adultas (Diptera: Sarcophagidae) são independentes do gene metalo beta lactamase

Moscas varejeiras (Diptera: Sarcophagidae) são causa conhecida de miíase e as bactérias de seus intestinos nunca foram estudadas quanto à atividade antibacteriana. Estudos antimicrobianos de Myroides spp restringem-se à cepas hospitalares. Uma bactéria Gram negativa, Myroides sp, foi isolada do intestino de moscas varejeiras adultas (Sarcophaga sp) e submetida à avaliação de parâmetros nutricionais pelo sistema BIOLOG GN, ao sequenciamento genético 16S rRNA, à sensibilidade a vários antimicrobianos pelo método de difusão de discos e à detecção dos genes de metalo beta lactamases (TUS/MUS). Os efeitos antagonistas foram testados contra bactérias Gram negativas e Gram positivas isoladas de material clínico humano, amostras ambientais e intestino do inseto. As espécies bacterianas incluíram Aeromonas hydrophila, A. culicicola, Morganella morganii subsp sibonii, Ochrobactrum anthropi, Weissella confusa, Escherichia coli, Ochrobactrum sp, Serratia sp, Kestersia sp, Ignatzschineria sp e Bacillus sp. A cepa Myroides sp foi resistente à penicilina G, eritromicina, estreptomicina, amicacina, canamicina, gentamicina, ampicilina, trimetoprim e tobramicina. Esta cepa apresentou atividade antimicrobiana contra todas as cepas exceto W.confusa, Ignatzschineria sp., A. hydrophila e M. morgani subsp sibonii. A resistência múltipla da cepa foi semelhante à de isolados clínicos, inibindo bactérias das amostras clínicas, ambientais e do intestino do inseto. Os genes de metalo beta lactamases (TUS/MUS) estavam ausentes, excluindo-se a resistência mediada por esses genes, o que indica o envolvimento de um mecanismo alternativo de secreção.

Palavras-chave: Mosca varejeira, *Myroides* sp, atividade antimicrobiana, metalo beta lactamase

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