Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic Marine waters

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Abstract: In the wake of the findings that Antarctic krills concentrate heavy metals at ppm level, (Yamamoto et al., 1987), the Antarctic waters from the Indian side were examined for the incidence of metal and antibiotic-resistant bacteria during the the austral summer (13th Indian Antarctic expedition) along the cruise track extending from 50°S and 18°E to 65°S and 30°E. The bacterial isolates from these waters showed varying degrees of resistance to antibiotics (Chloramphenicol, ampicillin, streptomycin, tetracycline and kanamycin) and metals (K_2CrO_4, $CdCl_2$, $ZnCl_2$ and $HgCl_2$) tested. Of the isolates screened, about 29% and 16% were resistant to 100ppm of cadmium and chromium salt respectively. Tolerance to lower concentration (10ppm) of mercury (Hg) was observed in 68% of the isolates. Depending on the antibiotics the isolates showed different percentage of resistance. Multiple drug and metal-resistance were observed. High incidence of resistance to both antibiotics and metals were common among the pigmented bacterial isolates. Increased resistance decreased the ability of bacteria to express enzymes. The results reiterate previous findings by other researchers that the waters of southern ocean may not be exempt from the spread of metal and antibiotic-resistance.

Key words: Antibiotic-resistance, metal-resistance, psychrotrophic bacteria, Antarctica

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Introduction:

Antarctic waters are considered relatively more pristine than the other oceanic waters experiencing anthropogenic influences only over larger time scales from the Northern hemisphere (Bonner, 1984). However, occurrence of high levels of heavy metals in krills (Yamamoto et al., 1987); Cd in caridean decapods (*Chorismus antarcticus* and *Notocrangon antarcticus*) which were highest among marine crustaceans (13 mg/kg dry weight) led to some reservation concerning the pristine nature of the Antarctic waters. Moreover, other crustaceans like amphipod *Maxilliphimedia longipes* and the isopod *Aega antarctica* (6-8 mg/kg) showed that they do not have requirements for reduced metals. Their requirements varied with interspecific heterogeneity (Petri and Zauke, 1993). Nygaerd et al. (2001) explained that low level of this metal even in higher organisms could be due to excretion of mercury through the growth of feathers or due to dilution during body growth. This led to the suggestion that in monitoring studies Antarctic organisms may not serve as the basis for global background levels. Sabry et al. (1997) observed antibiotic-resistance in metal tolerant marine bacterial population. An increase in the resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is due to the growth primarily of the resistant bacteria (Barkay and Olson, 1986; Muller et al., 2001, Rasmussen and Sorensen, 1998; 2001). Ecological studies have reported that metal and antibiotic resistance is becoming a global phenomena, with the Antarctic waters not being exempted, as the frequency of occurrence of plasmid-borne bacteria was high in Antarctic bacteria (Kobori et al. 1984). Plasmids are known to carry resistance to antibiotics and metals (Sobecky, 1999; Rasmussen and Sorensen, 1998; Smith et al., 1993). Hence the possibility of Antarctic bacteria to harbor antibiotic/metal resistance traits via horizontal transformation can be anticipated. Marine bacteria adsorb, accumulate and transform heavy metals (Chan and Dean, 1988) in most food chains. With the present rate at which some of the heavy metals bioaccumulate, (Krishnamurti and Nair, 1999; Guhathakurta and Kaviraj, 2000) including the Antarctic environment, it is pertinent to understand their effect at the primary microbial level especially in regions less affected by anthropogenic influences. Hence, Antarctic isolates were checked for their tolerance to heavy metals and drugs. In the light of these observations and in order

to appreciate how much the waters in these regions could contribute as source of heavy metals and drugs to higher trophic levels we examined the levels of metal resistance among Antarctic bacteria. Our results suggest that the Antarctic waters from the Indian side are not exempt to the spread of metal and antibiotic resistant bacteria and the enzymatic profiles of the isolates were apparently controlled to a certain extent by their resistance to metals.

Material and Methods

Water samples were collected along the cruise track extending from 50°S and 18°E to 65°S and 30°E during one of the Antarctic expeditions initiated by the Government of India from 1982 onwards. The details of sampling and analyses are described elsewhere (Loka Bharathi et al., 2001). Of the 185 isolates retrieved from ZoBell's medium 43 isolates which could survive repeated sub-culturing and showed psychrotrophic properties were used in this study. The isolates were identified based on taxonomic, physiological and biochemical properties detailed by Oliver and Smith, 1982.

Test for metal and drug resistance:

The test has been carried out as outlined in Nair et al. (1992). Briefly, working cultures were maintained in VNSS medium (Hermansson et al., 1987). Metal tolerance was examined on VNSS agar medium containing different concentrations of metals (10ppm of $HgCl_2$, 100ppm of $CdCl_2$, $ZnCl_2$ and K_2CrO_4). Similarly, the susceptibility tests to antibiotics (25 ppm chloramphenicol, 50 ppm ampicillin, 250 ppm streptomycin, 150 ppm tetracycline and 150 ppm kanamycin) were carried out in Muller agar. The activity of the enzymes oxidase, catalase, amylase, nitrate reductase, gelatinase, urease, phosphatase (organic and inorganic) (Kobori and Taga, 1978) and proteinase were recorded. The isolates were streaked on agar medium containing different substrates; for example for amylase activity 0.2% soluble starch, for lipase Tween 80 was used as substrate to test the enzymatic activity (Smibert and Krieg, 1981). The plates were incubated for a week at 4^0C.

Results:

Of the 43 cultures examined 68% showed tolerance to 10ppm of $HgCl_2$, 29% to 100 ppm of $CdCl_2$, 29% to 100 ppm $ZnCl_2$ and 16% to 100 ppm K_2CrO_4 (Table 1). Similarly all the tested isolates were resistant to ampicillin at 50 $\mu g.ml^{-1}$ while 63% to chloramphenicol at 25 $\mu g.ml^{-1}$ and streptomycin at 250 $\mu g.ml^{-1}$. Multiple resistance (5 antibiotics) was exhibited by 27% of the pigmented bacteria and 12% of the non-pigmented bacteria. Likewise, 36% of the pigmented isolates and 22% of the non-pigmented ones showed multiple resistance to metals. Thus, generally a higher percentage of pigmented bacterial isolates exhibited resistance to both antibiotics and metals (Fig. 1). Over 50% were positive for proteinase, and another 42% for gelatinase. The amylolytic strains accounted for only 21% (Table 2). Multiple metal-resistant bacteria could express less number of enzymes compared to single metal-resistance bacteria (Fig. 2). Among the 43 isolates 93% were gram negative and about 26% were pigmented. They belonged to nine genera - *Moraxella, Pseudomonas, Flavobacterium, Vibrio, Xanthomonas, Alcaligens, Micrococcus, Aeromonas* and *Acinetobacter*. The multiple resistant bacteria belonged either to *Flavobacterium* or *Alcaligenes* spp. (Table 2). However, *Aeromonas/Pseudomonas* and *Vibrio* were more versatile in their ability to elaborate 5-6 of the 9 enzymes studied.

Discussion:

Microorganisms undergo selection pressures in the presence of toxic compounds and develop resistance (Hideomi et al., 1977). The most common resistance is to metal and antibiotics, which can be a result of bio-essentiality or of abuse of the metal, and/or antibiotics. Enumeration of this resistant group from different geographical locations (Mudryk et al. 2000) has shown that these groups are ubiquitous. Residual effects of most of these heavy metals on aquatic biota are long lasting, as they can be non- available due to complex formation with organic matter. Thus they are not easily eliminated from these ecosystems (Forstner and Wittmann, 1979). In the Antarctic environment the low growth rates due to the temperature regime may promote high concentrations of some metals in certain organisms (Petri and Zauke, 1993). However, enumeration of these resistant

bacteria needs reservation as it not only depends on the nutritional status of the organisms (Brynhildsen et al., 1988) but also on the organic content of the medium used for enumeration (Nair et al., 1993). In this study, different heavy metals, which are known to be toxic viz., mercury, chromium, cadmium and a bioessential micronutrient zinc (Brynhildsen et al., 1988) which could be least toxic was used to find out the occurrence of resistant bacteria in Antarctic marine waters. This may be the reason for higher tolerance to Zn at 100 ppm. Bacteria are a potentially important source of metal accumulation in filter-feeding mollusk like limpets as ingestion of bacteria contributed up to 17% for Zn accumulation (Qiu et al., 2001). Interestingly, about 29% of the isolates were resistant to 100ppm of cadmium salt. This aspect is perhaps reflected at higher trophic levels too. The main reason for the high levels of cadmium observed in petrels and skuas was due to movement of Cd up the aquatic food chain as relatively high levels was noted in krill (Nygaerd et al., 2001). Chromate appeared to be more toxic than cadmium. Resistance was exhibited by only 16% of the isolates to 100 ppm K_2CrO_4. In the case of mercury the highest concentration of mercury resistance reported was for *E.coli* transformants, which grew in the presence of more than 40 ppm HgCl2 (Shiratori et al*.,* 1989). Recently De et al. (2003) isolated two strains from an area with intense shipping traffic, which grew on seawater nutrient agar solid medium with 75 ppm Hg. In general there is a sharp rise in resistant bacteria capable of tolerating very high concentration of metal mercury in the coastal environment of India and was irrespective of the current levels of pollution (Ramaiah and De, 2003). However, in this study resistance to lower concentration of Hg was observed in the Antarctic waters compared to the coastal waters suggesting that the contamination of this metal in these waters could be low. Many earlier studies observed that mercury resistant bacteria are also resistant to many antibiotics and other toxic chemicals (Barbieri et al., 1989; 1996; Canstein et al., 1999) by virtue of carrying plasmids and or transposons encoding genetically linked metal and antibiotic resistance.

The incidence of multiple resistance either to metal or antibiotics was observed in the Antarctic strains. Similar bacterial resistance to multiple heavy metals was reported from Providence river and the Narragansett bay (Traxler and Wood, 1981). Sabry et al. (1997)

showed that the response of the isolates to 11 tested antibiotics ranged from complete resistance to total sensitivity and multiple antibiotic resistance was exhibited by 70.4% of the total isolated population The highest incidence of metal-antibiotic double resistance existed between lead and all antibiotics (100%), copper and penicillin (95%) and nickel and ampicillin (83.3%).

Resistance to ampicillin was very pronounced in all our isolates, a phenomena reported from other areas (Zemelman et al., 1980). Transfer of virulence, conjugative, and/or antibiotic-resistance phenotypes may result in the increased dissemination of antibiotic-resistance and/or virulence determinants to bacteria in the environment. Mudryk et al. (2000) demonstrated the toxic effect of different concentrations of heavy metal on growth and respiratory activity of neustonic and planktonic bacteria, which were on the development and oxygen uptake in these bacteria and depended on the kind of the metal and its concentration. Our studies showed there was variation in the enzyme profile of the resistant isolates. Single resistance isolates had wide range of enzyme activity compared to multiple resistance isolates. Difference in enzymatic profile also have been observed in areas which are highly eutrophic compared to less eutrophic areas (Hoppe et al. 1998). However, the decreased versatility in expressing the different enzymes have to be reconfirmed with not only more isolates but also from other areas. Similarly the enzyme profiles of the resistant versus non-resistant isolates have to be studied to complement our observations.

While monitoring the Arabian Gulf, Chandy (1999) found heavy metal tolerance in chromogenic and non-chromogenic marine bacteria, whereas differential sensitivity to both metals and antibiotics were observed among pigmented and non-pigmented bacteria isolated from the coastal waters (Nair et al., 1992). Resistance to metal/antibiotics is also linked to chromogenesis as seen in our studies. In the Antarctic waters resistant bacteria were mainly gram negative. Tolerance to heavy metals was also reported to be pronounced in gram-negative bacteria (Nair et al., 1993; Duxbury, 1986). In the present work, all the strains of *Aeromonas* showed tolerance to mercury and chromium while there was difference in their resistance to antibiotics. A similar observation was made for

Pseudomonas, which also showed difference in resistance to metals. This may be due to the difference in strains. Beja et al. (2002) also suggested that considerable functional diversity might exist even in bacteria having similar taxonomic identity even at the molecular level. Ecological implication of resistant assemblages of Antarctic bacteria would mean better ability to adapt to changing systems

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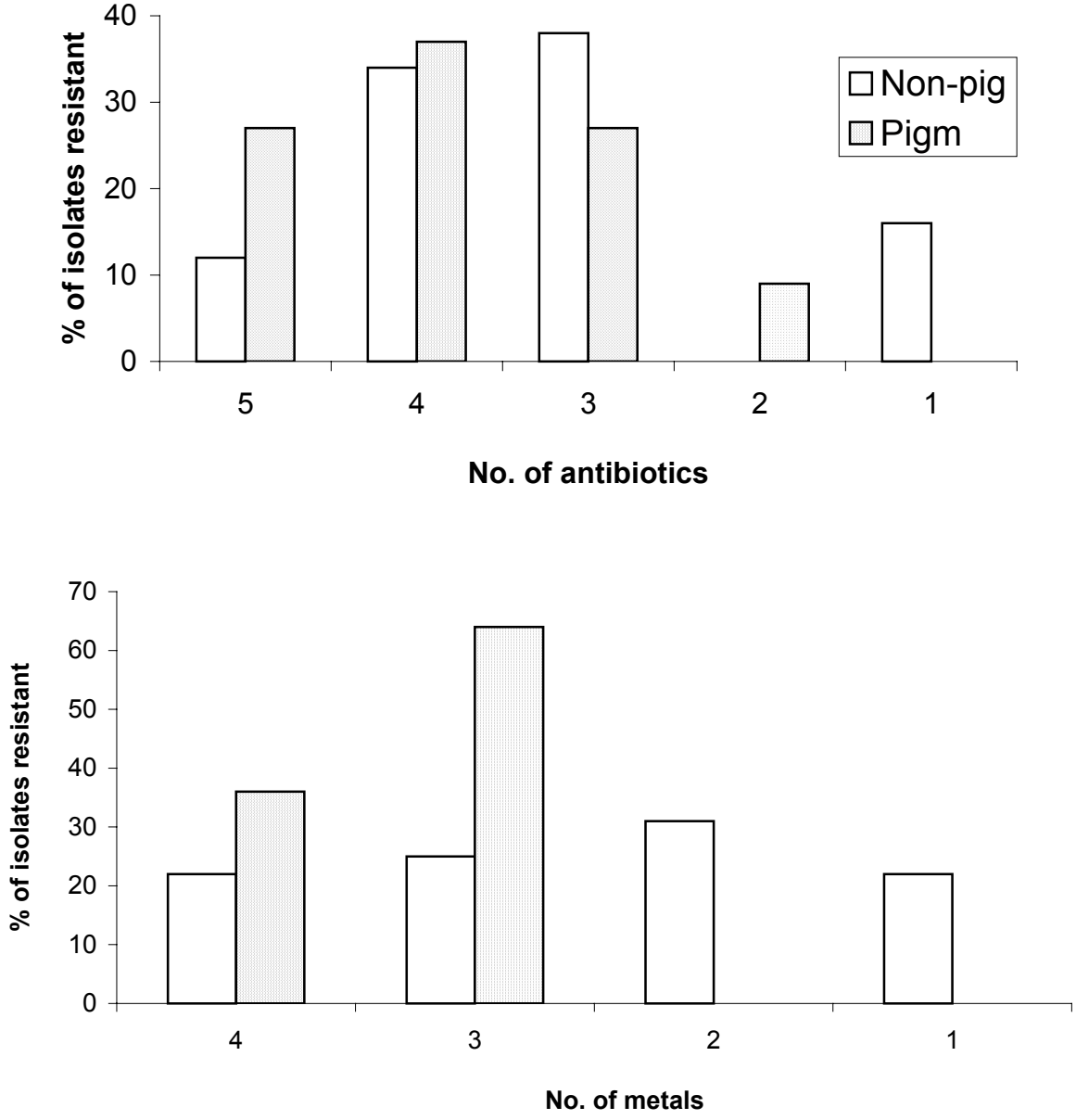

Fig. 1 Comparison of multiple resistance to antibiotic and metals in pigmented and non-pigmented bacterial isolates

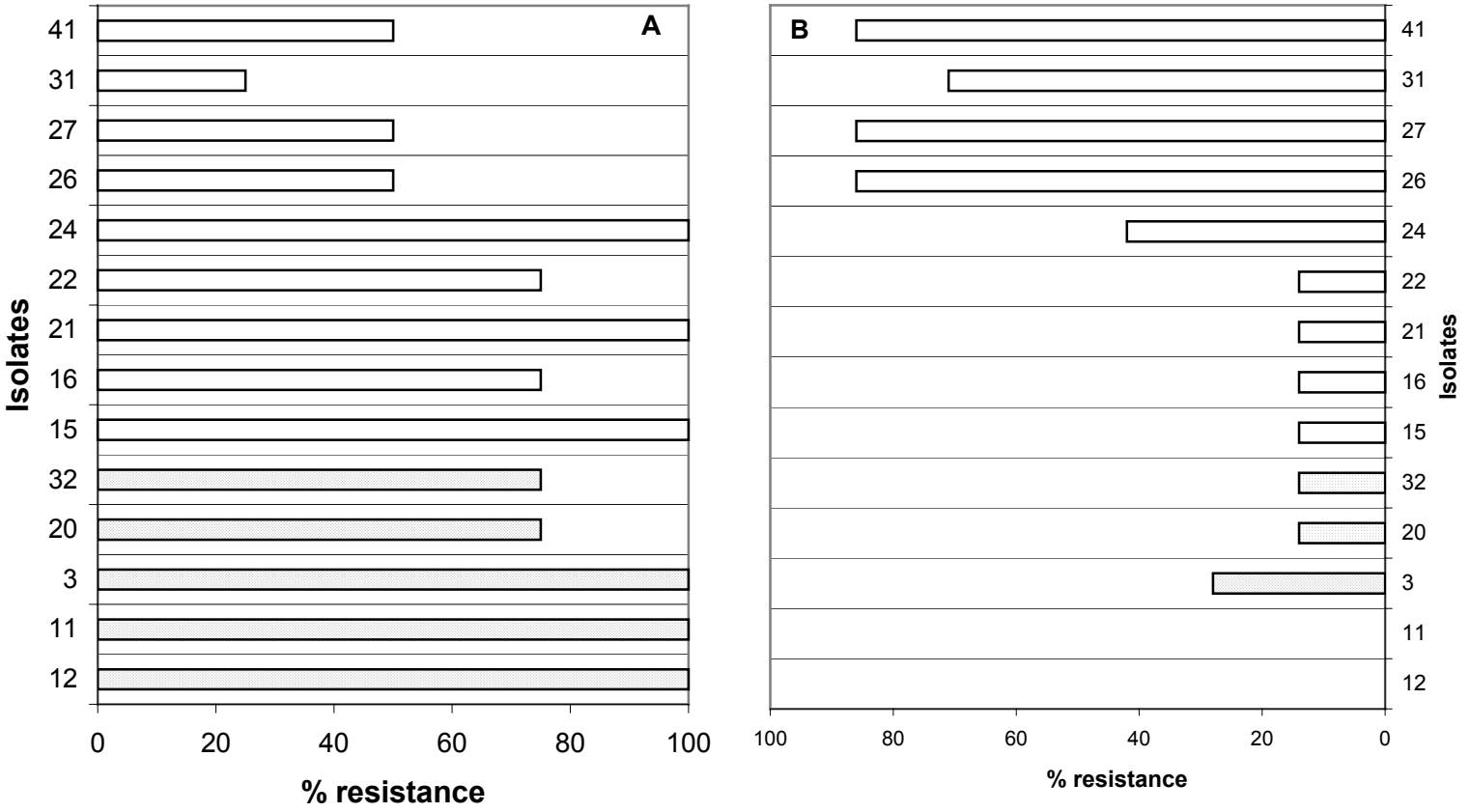

Fig. 2 Expression of multiple metal resistance (A) and multiple enzyme (B) in pigmented and nonpigmented isolates. Hatched bars indicate pigmented isolates.

Table 1. Percentage resistance to antibiotics and metals

	Conc (ppm)	**% Resistance**
Chloramphenicol	25	63
Ampicillin	50	100
Streptomycin	250	63
Tetracycline	150	11
Kanamycin	100	6
K_2CrO_4	100	16
$CdCl_2$	100	29
$ZnCl_2$	100	29
$HgCl_2$	10	68

Table 2. Expression for multiple antibiotic and metal resisitance and enzymatic activity in different genera

Isolate	Antibioti	Metal	Identification	Oxidase	Catalase	Amylase	NO_3 reductase	Gelatin-ase	Urease	Phosphatase Org	Phosphatase Inorg	Protease
Total	**5**	**4**										
1	4	3	*Moraxella*	✔	✔	✖	✖	✔	✔	✔	✔	✔
2	4	2	*Pseudomonas*	✔	✔	✖	✖	✖	✔	✖	✖	✖
(3)	**5**	**4**	***Flavobacterium***	✔	✔	✖	✖	✖	✔	✖	✖	✔
4	4	3	*Vibrio*	✔	✔	✖	✔	✔	✔	✔	✖	✖
(5)	2	3	*Flavobacterium*	✔	✔	✖	✖	✔	✔	✔	✖	✔
6	4	1	*Moraxella*	✔	✔	✖	✖	✖	✔	✖	✖	✖
7	5	2	*Moraxella*	✔	✔	✔	✔	✖	✖	✖	✔	✖
8	**5**	**4**	***Alcaligenes***	✖	✔	✖	✖	✔	✖	✖	✖	✖
(9)	4	3	*Flavobacterium*	✖	✔	✖	✔	✖	✔	✔	✔	✖
10	3	3	*Alcaligenes*	✔	✔	✔	✔	✖	✔	✔	✔	✖
(11)	4	4	*Flavobacterium*	✖	✔	✖	✖	✖	✖	✖	✖	✖
(12)	**5**	**4**	***Flavobacterium***	✖	✔	✖	✔	✖	✔	✖	✖	✖
13	4	2	*Alcaligenes*	✔	✔	✖	✖	✔	✖	✖	✖	✔
14	3	2	*Alcaligenes*	✔	✔	✖	✖	✔	✖	✖	✖	✔
15	3	4	*Pseudomonas*	✔	✔	✖	✖	✖	✖	✔	✖	✖
16	3	3	*Pseudomonas*	✔	✔	✖	✖	✖	✖	✔	✖	✖
17	3	3	*Pseudomonas*	✔	✔	✖	✖	✖	✖	✔	✖	✖
18	4	4	*Pseudomonas*	✔	✔	✖	✖	✖	✔	✖	✔	✖
19	3	3	*Micrococcus*	✖	✔	✖	✔	✖	✖	✖	✔	✖
(20)	3	3	*Flavobacterium*	✖	✔	✖	✔	✖	✔	✖	✖	✖
21	4	4	*Pseudomonas*	✔	✔	✖	✖	✖	✖	✔	✖	✖
22	4	3	*Moraxella*	✔	✔	✖	✖	✖	✔	✔	✖	✔
23	5	2	*Alcaligenes*	✔	✔	✖	✖	✖	✖	✔	✖	✔
24	3	4	*Micrococcus*	✔	✔	✖	✖	✔	✖	✔	✖	✔
25	3	4	*Micrococcus*	✔	✔	✖	✔	✔	✖	✖	✖	✔
26	3	2	*Alcaligenes*	✔	✔	✖	✔	✔	✔	✔	✔	✔
27	4	2	*Aeromonas*	✔	✔	✔	✔	✔	✔	✔	✖	✔
28	4	4	*Pseudomonas*	✔	✔	✖	✔	✖	✔	✖	✖	✔
29	3	3	*Vibrio*	✔	✔	✔	✔	✔	✔	✔	✖	✔
30	3	1	*Pseudomonas*	✔	✔	✖	✔	✔	✖	✔	✖	✔
31	3	1	*Pseudomonas*	✔	✔	✖	✔	✔	✖	✔	✔	✔
(32)	5	3	*Xanthomonas*	✖	✔	✖	✔	✖	✖	✖	✖	✖
(33)	3	3	*Flavobacterium*	✖	✔	✖	✖	✖	✖	✔	✖	✔
34	4	3	*Pseudomonas*	✖	✔	✖	✖	✔	✖	✖	✖	✖
35	3	1	*Pseudomonas*	✖	✔	✖	✖	✖	✖	✖	✔	✔
(36)	3	3	*Flavobacterium*	✖	✔	✖	✔	✖	✖	✖	✖	✔
37	4	3	*Acinetobacter*	✖	✔	✖	✖	✔	✖	✖	✔	✔
(38)	4	4	*Flavobacterium*	✔	✔	✖	✖	✖	✖	✔	✖	✖
39	**5**	**4**	***Alcaligens***	✖	✔	✖	✖	✔	✖	✔	✖	✔
40	3	2	*Acinetobacter*	✖	✔	✖	✖	✖	✖	✖	✖	✔
41	1	2	*Pseudomonas*	✔	✔	✔	✔	✖	✖	✔	✔	✔
(42)	4	3	*Flavobacterium*	✖	✔	✖	✔	✔	✖	✔	✖	✔
43	5	2	*Aeromonas*	✔	✔	✔	✔	✔	✖	✔	✖	✔

( ) Pigmented isolates