ANTAGONISTIC BACTERIA FROM LIVE CORALS, TUTICORIN COASTAL WATERS, SOUTHEASTERN INDIA

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ABSTRACT

The objective of this study is to isolate and production of secondary metabolites with bioactive substances by coral reef (*Acropora formosa* and *Favia palida*) associated bacteria was carried out from the Tuticorin coastal waters, Southeastern India. The isolated coral associated bacteria were found to have an antagonistic effect against 10 human pathogens. The pathogens were *Escherichia coli*, *Shigella dysentriae*, *Staphylococcus epidermidis*, S. *aureus*, *Klebsiella pneumoniae*, *Pseudomonas aerogenosa*, *Vibrio cholerae*, *Streptococcus pneumoniae*, *S. faecalis* and *Bacillus cereus*. It was observed that, out of the total 689 bacterial strains isolated, 10 and 13% of isolates from *A. formosa* and *Favia palida*, respectively were found to have antagonistic activity against the pathogens, A higher percentage of antagonistic strains were conferred from *Favia palida* with 13%. It was observed that all antagonistic strains were able to inhibit at least two of the human pathogens.

Keywords: Antagonistic bacteria, human pathogens, corals, Tuticorin.

INTRODUCTION

Antibiotics are defined as chemical substances fashioned by microorganisms and they have a major impact on the development of medical science Microorganisms not only cause infection but also produce organic compounds that can treat a variety of infectious disease (Chellaram *et al*., 2009). There is no doubt that the discovery of antibiotic drugs revolutionized the world of medicine. However, even after five years human beings are far from winning the battle against infectious disease. In particular, novel secondary metabolites, including antibiotics from marine bacteria are attracting attention because of the growing demand for new antibiotics (Levy, 1998).

Marine microorganisms are of considerable current interest as a new and promising source of biologically active compounds. They produce a variety of metabolites, some of which can be used for drug development (Chellaram *et al*., 2010 and Grossart *et al*., 2004). Recently, it was shown that some bioactive compounds isolated from invertebrates originate from symbiotic microorganisms e.g., Tetrodotoxin, Saxitoxin, Okadaic acid, Surugatoxins, 2,4-diacetylphloroglucinol produced etc. (Isnansetyo *et al*., 2003). Association between marine invertebrates and symbiotic bacteria are increasingly recognized as widespread and of biological importance (McDonald, *et al*., 1996). Scanty works reporting the occurrence of bacteria associated with marine fauna, in particular, the corals (Santavy, 1995). Koh (1997) had analyzed over 100 species of stony corals and found them to produce antimicrobial compounds. Thus, corals were found to produce and be endowed with their own microbial defenses, but the strains which inhabited on their surface were found to be producing their own defenses. A few studies have suggested that coral may associate with specific microbes. Santavy (1995) observed that *Porites astreoides* samples collected from throughout the Caribbean harbored bacteria-filled ovoid. It has also been shown that some corals harbor nitrogen-fixing microbes to obtain fixed nitrogen from associated microbes that are fed and protected in an anaerobic environment within the colony.

Microbial natural products remain one of the most important sources of lead compounds for the pharmaceutical industry. Despite a shift towards alternative sources such as synthetic combinatorial libraries, computer-based molecular modeling and most recently, combinatorial biology or the manipulation of biosynthetic gene clusters, the pharmaceutical pipeline remains filled with traditional, microbial derived natural products or their derivatives. It is hard to deny that with 3.5 billion years of biosynthetic experience, microorganisms remain nature's best chemists and along with this accomplishment, one of the best sources of novel, biologically active organic molecules from which to build a drug discovery program (Jensen *et al*., 2003). Recent studies have shown that these antibacterial compounds are not only inhibiting the human pathogens but also fish pathogens (Strahl *et al*., 2002) Hence, the present investigation was undertaken to isolate the coral

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reef (Stag horn: *Acopora formosa* and Mass: *Favia palida*) associated bacteria for antagonistic effect against human pathogens using well diffusion method *in vitro*.

MATERIALS AND METHODS

Isolation of the surface associated bacteria from corals

The coral associated bacteria were obtained during by swabbing a tiny area (1cm^2 in triplicates) of the external surface of live scleractinian corals such as *Acropora formosa* and *Favia palida* by SCUBA diving from the open sea of the Tuticorin (Lat 8º45 and Long 78º13'E), Southeastern India. Then the swabs were placed in sterile plastic bags, brought to the laboratory and transferred to 9 ml of sterile seawater and vortexed. The swab samples were serially diluted and plated on Zobell Marine 2216 E agar (ZMA) using pour plate method. For incubation, the plates were inverted in sealed sleeves in the dark at the room temperature of 20-25^{o}C for 5-7 days. Morphologically different colonies were selected randomly. The number of Gram positive and negative colonies and pigmented and non-pigmented colonies was noted. Axenic culture were obtained by streaking and re-streaking on ZMA plates and subsequently stored as ZMA stab culture at 4^{o}C. Gram staining was carried out for all the isolated strains.

Screening of the isolates for antagonistic activity against human pathogens

The coral associated isolates were tested for antagonistic effect by double agar overlay method (Dopazo *et al*., 1988) against ten selected human pathogens. Ten numbers of each pathogen were used for antagonistic effect study. The pathogens were *Escherichia coli* (ATCC 25922), *Shigella dysentriae* (ATCC 13313), *Staphylococcus epidermidis* (ATCC 12228), S. *aureus* (ATCC 29737), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aerogenosa* (ATCC 10197), *Vibrio cholerae* (ATCC 14100), *Streptococcus pneumoniae* (10015), *S. faecalis* (10741) and *Bacillus cereus* (ATCC 10876). All the pathogens were obtained from Christian Medical College (CMC), Vellore. The isolates were grown on yeast extract peptone (YEP) medium (75% sea water) plates for three or more transfers before using them in the screening assays. All isolates were tested for the production of antibacterial metabolites using Double agar overlay method. The 18 hours old isolates were spotted on the 50% ZMA and incubated at room temperature for 12 hour. All pathogens were cultured in Tryptone Soya Broth (TSB) and the 12-18 hours old cultures were used for the experiments. About 20 µL of the test cultures were suspended in 20 ml of soft Tryptone Soya Agar (TSA) and were poured immediately over the colonies of the antagonistic marine bacteria on the ZMA plates. The plates were incubated at 29^{o}C for 24 hours. The cleared zone around the macro-colonies of the antagonistic bacteria was measured and the radius of zone of inhibition was noted in mm.

RESULTS

Isolation of the coral reef associated bacteria

A total of coral surface associated 689 bacterial strains were isolated. Among that 354 and 335 strains were from *Acropora formosa* and *Favia palida* respectively. The number of Gram positive, Gram negative, pigmented and

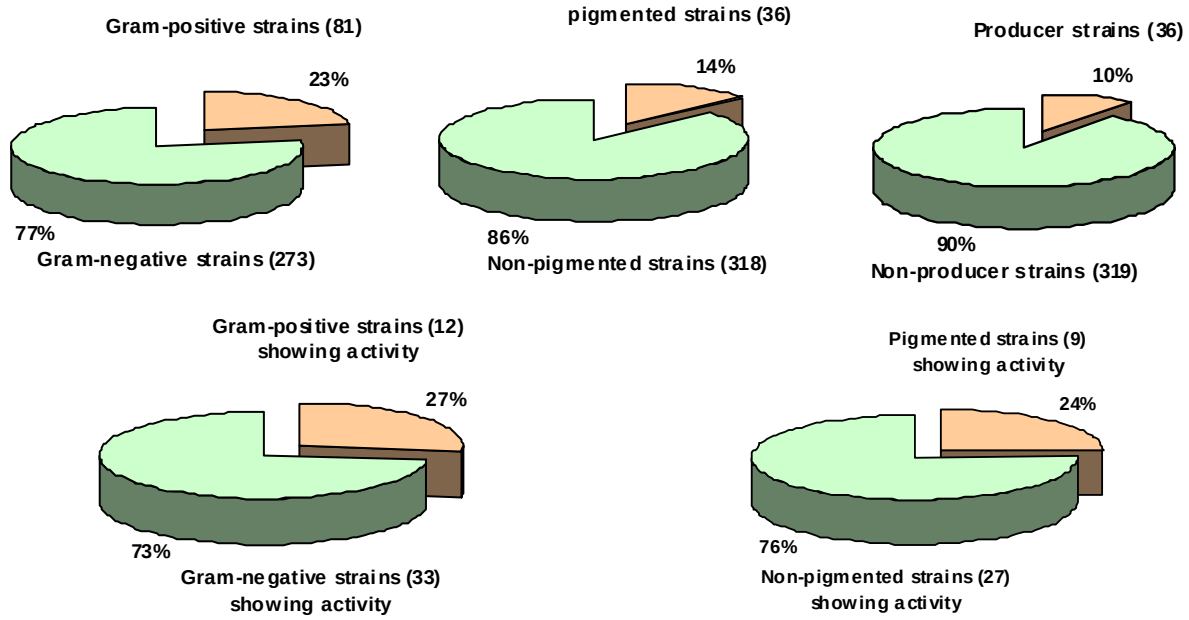


Fig. 1: Bacterial strains isolated from surface of *Acropora formosa* and antagonistic effect against human pathogens.

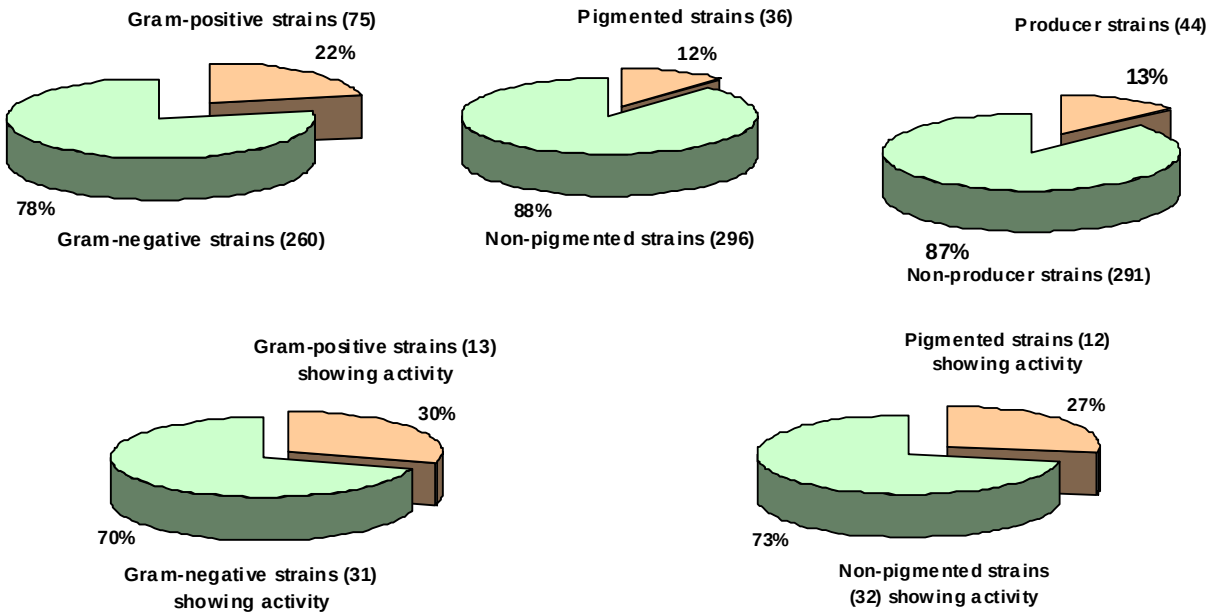


Fig. 2: Bacterial strains isolated from surface of *Favia palida* and antagonistic effect against human pathogens.

non-pigmented strains was isolated from 2 coral species (figs. 1 and 2). The majority of the pigmented colonies were yellow, red, brown, orange and black in color.

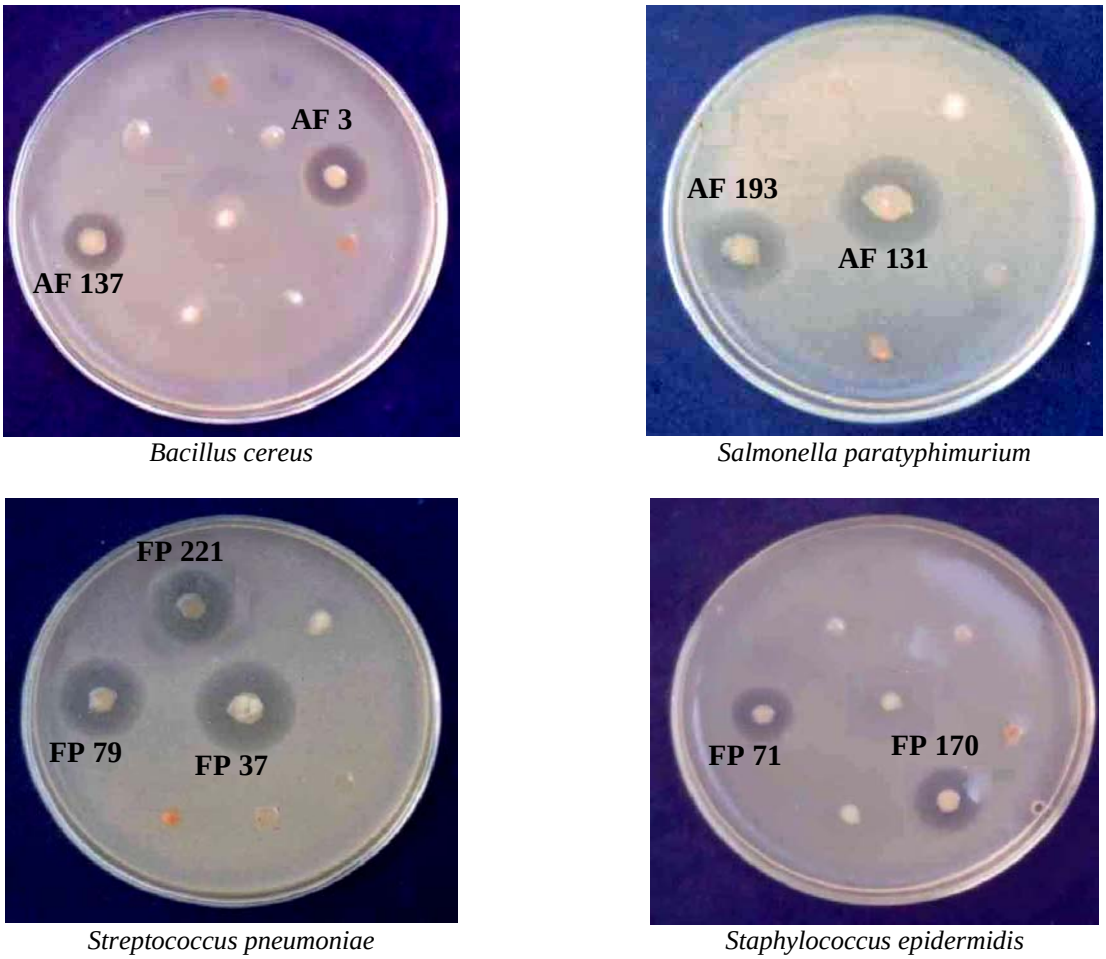
Antagonistic activity of the coral reef associated bacteria against human pathogens

Among the 689 bacterial strains, only 10 and 13 percentages of isolates from *A. formosa* and *Favia palida* respectively were found to have antagonistic activity against the pathogens (figs. 1 and 2). A higher percentage of antagonistic strains were conferred from *Favia palida* with 13%. The results indicate that the higher percentage (27) of the producer strains was found to be pigmented were isolated from *Favia palida*. It was observed that all antagonistic strains were able to inhibit at least two of the pathogens.

The antagonistic bacteria of corals, *A. formosa* and *Favia palida*, against the ten human pathogens are shown in tables 1 and 2. It was observed that the AF131 and AF193 inhibited the growth of *Vibrio cholerae* to above 6.5 mm and strains FP37, FP79 and FP221 inhibited the growth of *Streptococcus pneumoniae* to 6.5 mm, 5.5 mm and 5 mm respectively (fig. 3). Coral mucus has been reported to be an important fraction of reef detrital contains energy-rich lipid compound, while most reef detritus is nitrogen-poor. This suggests that, as a part of reef detritus mucus may be a valuable source of nutrients and energy for detritus and suspension feeders. The mucus film covering living corals harbor microorganisms which are adapted to several reports for existence in such habitat (Meikle *et al*., 1988). Marine microbial natural products still appear as the most promising source of the future antibiotics that society is expecting. The arguments supporting this idea are the unparalleled structural diversity that can be found in nature, the fact that natural antibiotics have apparently been shaped by evolution to make them effective to killing microorganisms and the suggestions that the field still unexplored is huge. The rich variety of chemically novel and biologically active metabolites serves to indicate that marine bacteria are a genetically rich resource for recombinant technologies (Moore, 1999).

DISCUSSION

In the present investigation, a total of 660 strains of surface associated bacteria were isolated from two species of scleractinian corals, which were tested for antibiotic production. Of these non-pigmented strains (86-90%) were higher in number from both species than the pigmented ones (10 – 14 %). This observation is on par with the findings of Jeyasekaran *et al*. (2002), who have reported that pigmented bacterial flora was lower by about 2-3 log counts than the total culturable bacterial flora observed in the marine samples from seawater, sediments, sea plants and bivalves. The finding that the percentage of Gram positive strains was found to be lower (20-27%) than the Gram negative strains (73-80%) agrees with Fenical (1993) who reported that the bacteria present in seawater were mainly negative rods. Gnanambal *et al*. (2005) observed that, 61% were identified as Gram negative strains from gorgonid corals, *Subergorgia suberosa* and *Junceella juncea*. Another study revealed that the bacterial strains isolated from various regimens of the marine environment showed that 82.28 % were Gram negative (Strahl *et al*., 2002).



Bacillus cereus *Salmonella paratyphimurium*

Streptococcus pneumoniae *Staphylococcus epidermidis*

Fig. 3: Antibacterial activity of coral reef associated bacteria against some pathogens.

Study by Chelossi *et al*. (2004) deals similar aspects and their findings imply that 58% of the aerobic heterotrophic bacterial strains isolated from the sponge, *Petrosia ficiformis* were identified as Gram negative.

Inhibition zones of up to 22 mm were observed for 3 coral species against human pathogens as reported by Jeyasekaran *et al*. (2002). There are only a few reports pertaining to the study of coral reef associated bacteria, (Gast *et al*., 1998). In the present study, the percentage of bacterial isolates of corals antagonistic to human pathogenic bacteria was found to be 10% (*A. formosa*) and 8% (*Favte abdita)*. This result trends along with the report of Nair and Simidu (1987) who stated that 8.8% out of the 45 epibiotic bacteria isolated from different marine samples displayed anti-staphylococcus activity. The epiphytic bacterial strains isolated from the inter-tidal seaweeds showed inhibitory activity against *Vibrio harveyi, V. anguillarium* and *A. hydrpohila* (Lemos *et al*., 1985).

In this study, 13-18% of the producer strains were isolated and from this 70% were non-pigmented which contradicts the findings of Rosenfeld and Zobell (1947) that reported that most of the antibiotic-producing marine bacteria were pigmented. However, a smaller percentage of antagonistic strains (30%) were found to be pigmented which might be due to the reason that pigments have been associated with antibacterial activity as is the case for cyanobacteria (Lemos *et al*., 1985). Pigmented bacteria are also known to be potential antibiotic producers as reported by Shiba and Taga (1980). One of the findings of the present study is that Gram negative strains (73-80%) showed comparatively higher antagonism against the test strains that the Gram-positive ones (20-27%) which deviates from the works of Fenical (1993).

Among the producer strains, AF131 (6.5 mm), AF193 (6 mm) and FP37 (6.5 mm) were found to be have maximum inhibition zone against human pathogens. Similar zones of inhibition by marine antagonistic bacteria were reported by earlier workers (Patil *et al*., 2001). Marine microbes have a higher possibility of yielding natural products with unprecedented and interesting bioactivity. The antagonistic marine bacteria isolated from the corals may produce antibacterial potential compounds with novel structures which can be explored to generate pronounced biological activity in the future.

Table 2: *Favia pallida* associated bacteria exhibiting antagonistic activity against human pathogens

Pathogens	*E. coli*	*Shigella dysentriae*	*Staph. epidermidis*	*Staph. aureus*	*Klebsiella pneumonia*	*Pseud. aerogenosa*	*Vibrio cholerae*	*Strep. pneumonia*	*Strep. faecalis*	*B. cereus*
Isolates	Radius of the zone of inhibition (mm)									
FP1	T	1.5	T	2	T	T	T	T	-	T
FP9	1.5	2	2	1.5	T	2.5	4	3	2.5	4.5
FP19	T	-	-	T	T	1.5	2	1.5	T	3.5
FP23	2.5	2	3	2	1.5	T	4.5	5	4	2.5
FP37	3	3.5	3	4	4.5	3	4.5	6.5	2.5	3.5
FP41	T	-	2	1.5	T	-	-	T	1.5	T
FP47	3.5	4	3	4.5	3	4	4.5	3	2.5	3.5
FP71	4.5	4	4	2.5	2	3.5	2	3.5	4.5	3
FP79	3.5	2.5	4	4.5	4	4.5	4.5	5	4	5
FP87	6.5	6	5	5.5	5.5	4.5	6	5.5	5	5
FP93	T	-	-	T	1.5	2	T	-	1.5	T
FP99	-	-	T	T	1.5	2	-	T	1.5	-
FP106	4.5	3.5	2.5	2	1.5	3	5	4	3	2.5
FP117	T	-	-	-	T	1.5	2.5	T	2	T
FP131	-	T	T	T	T	T	2	-	-	T
FP148	1.5	T	T	1.5	T	-	2	T	T	-
FP159	3	4.5	2	2	2.5	3.5	5	4.5	4.5	5
FP167	1.5	2	2	3	T		-	1.5	T	1.5
FP170	3.5	4	5	4	3.5	2.5	4	4.5	3.5	4
FP188	4	4.5	3	3	3.5	3.5	4	4.5	5	4
FP190	3.5	4	3	3.5	3.5	3	4.5	1.5	2.5	3.5
FP199	4	3	2.5	4	4	4.5	4	3.5	4.5	4
FP210	3.5	2	2	2.5	2	1.5	3.5	3	4.5	4.5
FP215	3	2.5	3.5	4	3	3.5	T	1.5	2.5	2
FP221	2.5	2	4	3	2	2.5	3.5	4.5	5	5
FP228	T	-	T	T	-	-	-	-	2	-
FP231	2.5	3	2	2.5	3	3.5	3	3	2.5	2
FP239	1.5	2	2.5	2	2.5	3	2.5	2	2.5	1.5
FP240	3	4	4.5	3.5	3	2.5	3.5	2.5	2	3
FP245	5	4	4.5	4	3.5	3.5	3	2.5	4	4.5
FP249	T	1.5	2	T	-	1.5	2	T	1.5	2.5
FP251	2	2.5	3	2.5	1.5	2.5	1.5	2	3	2
FP259	T	2	1.5	2.5	1.5	T	2	2.5	T	1.5
FP269	3	3.5	4	3	3.5	4	3.5	4.5	4	5
FP274	2.5	3	2	3	3.5	2.5	2	3	3.5	2.5
FP281	T	2	T	1.5	-	T	1.5	-	T	1.5
FP287	2.5	3	3	2.5	3.5	4	2.5	3	3.5	2
FP288	3	35	4	4.5	5	4.5	5	4	3.5	4
FP290	T	-	2	1.5	T	1.5	2	T	1.5	T
FP294	2	2.5	1.5	3	2.5	2	1.5	T	2	2.5
FP301	T	1.5	2	T	-	1.5	T	1.5	1.5	T
FP310	3	3.5	2.5	3	2.5	2	1.5	2.5	2	3
FP321	1.5	-	T	1.5	2	-	-	T	1.5	2
FP329	4	3.5	2.5	3.5	3	4	4.5	3.5	3	2.5

T- Trace, - Nil

Table 1: *Acropora formosa* associated bacteria exhibiting antagonistic activity against human pathogens

Pathogens	*E. coli*	*Shigella dysentriae*	*Staph. epidermidis*	*Staph. aureus*	*Klebsiella pneumonia*	*Pseud. aerogenosa*	*Vibrio cholerae*	*Strep. pneumonia*	*Strep. faecalis*	*B. cereus*
Isolates	Radius of the zone of inhibition (mm)									
AF3	5	4	6	3	2.5	-	4	3	3.5	-
AF7	4.5	5.5	3.5	3	2	T	5	3	2.5	5
AF17	T	T	1.5	2	2.5	1.5	T	-	T	T
AF23	3.5	3	T	-	1.2	2.5	2.5	T	-	3.5
AF25	T	1.5	T	-	-	-	2	2.5	T	T
AF34	6	6.5	8	7	8	3.5	3.5	6	5	2.5
AF61	3	2	-	-	-	T	T	2.5	-	T
AF77	-	-	T	-	T	T	-	-	2.5	T
AF88	3	3.5	2.5	4	4.5	5	5	6	5.5	4
AF101	4.5	3.5	5	5.5	4	4	3.5	5	4	4.5
AF112	3	2	2.5	1.5	T	2	T	T	3.5	2.5
AF120	2	3	3.5	2	2.5	T	3	3.5	4.5	3
AF131	3.5	3	4.5	2.5	3	T	6.5	5.5	4.5	4.5
AF137	4.5	4	5.5	3	3	3.5	5.5	5	5	5
AF143	5	3	4	3	4	T	-	T	4	T
AF152	2	2	1.5	T	T	-	2	1.5	2	2.5
AF159	T	-	T	1.5	2	-	T		-	T
AF170	3	2.5	3.5	4	3	2.5	4	4.5	3.5	2.5
AF178	3.5	3	2.5	3	3.5	1.5	3.5	3	2.5	4.5
AF181	T	2	-	-	T	1.5	2	T	1.5	2.5
AF188	-	-	-	T	-	1.5	-	3	T	1.5
AF189	5	6	6.5	5	4	4.5	6	6.5	T	5.5
AF193	2	2.5	3.5	3	4	4.5	6.5	4	5.5	4
AF199	T	-	T	T	2	T	-	-	3	T
AF210	2.5	2	2.5	2	2	1.5	2	1.5	2	2
AF223	2	1.5	T	3	2.5	-	3	2.5	2	2
AF229	3	1.5	-	T	-	1.5	2.5	2	1.5	3
AF238	2	2.5	1.5	3	1.5	-	T	3	2.5	1.5
AF239	3	4	3.5	4.5	4	5	3	2	2.5	1.5
AF243	4	5	5	5.5	6	4.5	5	5.5	4.5	5.5
AF250	5	4	5	6	5	4.5	3.5	5.5	5	6
AF253	4.5	4.5	5	4	3.5	3	4	5	4.5	5
AF262	3	2.5	2	T	1.5	3	2.5	2	2	T
AF267	2.5	2	2.5	3	3.5	3	2.5	3	3	2
AF289	-	1.5	T	T	T	1.5	2	-	1.5	2
AF291	2	3	2.5	3	2	2.5	1.5	2	2.5	T
AF302	1.5	2	2	2.5	1.5	T	-	2	1.5	T
AF314	T	-	2	1.5	T	2	2.5	2	1.5	-
AF321	3.5	2	2.5	1.5	3	3	2	1.5	2	3
AF330	2.5	3	3.5	4	3.5	4	4.5	3.5	3	3.5
AF339	2	2.5	3	2.5	1.5	2.5	2	3	1.5	T
AF341	1.5	-	-	T	2	1.5	-	T	T	1.5
AF346	1.5	1.5	2	T	1.5	-	T	1.5	2	1.5
AF349	-	T	1.5	2	-	T	1.5	2	2	1.5
AF351	2	2	3	3.5	2.5	3.5	2	2.5	1.5	3

T- Trace, - Nil

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