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Synergistic Activity of Antibiotics and *Trachyspermum ammi* (Ajwain) Extract on MDR *Staphylococcus aureus*

KEYWORDS: Multidrug resistance, Trachyspermum ammi, herb extracts, Staphylococcus aureus.

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ABSTRACT *New emerging trends in multidrug resistance among several groups of microorganisms against different classes of antibiotics led to development of different strategies. Total 106 Clinical samples were screened for isolation of Staphylococcus sp. Total 21 suspected isolates of Staphylococcus aureus were identified on the basis of morphological, cultural & biochemical characteristics and all of them were found to be Staphylococcus aureus. 17 isolates were associated with pus samples, 3 with urinary tract infection (UTI) and 1 was associated with blood. Antibiogram study of these isolates revealed that all these isolates are resistant to several antibiotics out of 16 antibiotics tested. Synergistic study of extracts of T. ammi and antibiotics on susceptibility of resistant Staphylococcus isolates showed that most of the extracts exhibited potentiating effect on antibiotics. The potency shown by these extracts may explore their use against multidrug resistant microorganisms in combination therapy.*

Introduction:

Natural medicine is the chemical agents derived from Natural origin that is used for curative purpose (Dorman H.J. and Deans S.G., 2000). Medicinal plants are natural resources for valuable products that can be used in the treatment of various ailments. In Indian system of medicine, (*T. ammi*) ajwain is most often used herb and administered for stomach disorders, a paste of crushed herb were applied externally for relieving local pains; and a hot and dry concentrates of herb were also layered on the chest to cure asthma. The synergistic and additive effect of phytochemicals in combination play key role to use plant extracts as antimicrobial agents. Aqueous extract of *T. ammi* is a popular preparation for diarrhea, stomach ache, carminative, expectorant, antiseptic, amoebiasis and antimicrobial applications. Some extended use of *T. ammi* also cures abdominal tumor and piles (Krishnamoorthy V and Madalageri MB., 1999). Problem of comfort dipsomania, hysteria, relieving flatulence, dyspepsia, spasmodic disorders, flatulence, common cold, acute pharyngitis, infections with worms and congested throat can be managed with ajwain formulation (Ranjan B *et. al.,* 2011). The mode of action is due to inhibition of microbial growth and oxidation & hindrance of some enzymatic reactions. In addition *T. ammi* and other spices offer a promising alternative for food safety (Arora D.S. and Kaur G.J., 2007). Some studies claim that the phenolic compounds present in spices and herbs might play a major role in their antimicrobial effects (Hara-Kudo Y., *et. al., 2004* and Horace D.G., 1982).

The spread of multi-drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases. An alarming increase in bacterial strains resistant to existing antimicrobial agent population seeks a new effective treatment against pathogens. Over the time, spices have evoked interest in natural products to heal many infectious diseases (Parekh *et al.,* 2005). Spices are some of the most commonly used natural antimicrobial agents in foods (Hirasa and Takemasa, 1998; Nevas *et al.,* 2004) and spices are the common dietary adjuncts which contribute to the taste, flavor and aroma to foods as well as a stop signal to stabilize the foods from the microbial deterioration (Kizil and Sogut, 2003). Natural compounds in spices were found to possess antimicrobial activity (Shelef 1983). Spices plays key role in the food spoilage and effects of these natural compounds in combination and or as crude have shown to limit food-spoilage micro-organisms (Mandalari G. *et. al.,* 2007 and Lai P.K. and Roy J., 2004). Essential oils and extracts of certain plants have been proved to have antimicrobial effects (Burt S., 2004). Spices are rich source of biologically active antimicrobial compounds which are effective against treatment of both Gram positive and Gram negative bacterial strains (Lia and Roy, 2004; Russel, 1991).

Bacteria have the genetic ability to transmit and acquire resistance to several drugs which is called as multi drug resistant (MDR) pathogens (Soulsby J., 2005). Resistance to drugs is higher in developing countries than developed one because of extensive and indiscriminate use of antibiotics (Akram M., 2007) and people's ability to self-medicate without a prescription from a physician. Among the wide range, beta (β)-lactams are the most varied and widely used antibiotics (Bronson JJ and Barrett JF., 2007) and its resistance due to production of β-lactamases which degrade the basic structure of (β)-lactum. A property production of β-lactamases has been attributed to the spread of plasmid-mediated extended spectrum β-lactamases (ESBLs) (Sanders CC and Sanders WE Jr., 1987). Antibacterial activity of spices against several species of bacteria have been demonstrated, which includes some serotypes of *Salmonella*, such as *S. typhimurium* (Lachowicz *et al.,* 1998; El-Gayyar *et al.,* 2001) *S. enteritidis*, *S. infantis* (Alzoreky and Nakahara, 2002), and *S. anatum* (Swetwiwathana *et al.,* 1999).

There are considerable variations in resistance of different microorganism to a same spice and for the same microorganism to different spices (Arora and Kaur, 1999). Seeds of *T. ammi* (ajwain) contain phyto-chemicals such as pinene, cymene, limonene and terpinene. In past practices volatile oils has been suggested to either inhaled or applied topically over the skin, which act by means of their lipophilic fraction reacting with the lipid parts of the cell membranes, and modify the activity of calcium ion channels (Buchbauer G and Jirovetz L., 1994). The presence of

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terpenes, glycosides and sterols in plant has been found to be responsible for anti-inflammatory properties whereas, the phenolic constituents of ajwain are mainly responsible for the antiseptic and antitussive properties (Ray B., 1996 and Brull S. and Coote P., 1999).

The current study was carried out to investigate the bactericidal and synergistic effect of *T. ammi* extracts on antibacterial efficacy of antibiotics often used to treat MDR pathogens.

Material and Methods:

Collection of clinical samples:

Clinical samples of urine, pus, blood and sputum sample were collected from different pathology laboratories of Nagpur (MS), India.

Isolation of *Staphylococcus sp.* from various clinical Samples:

A sample was immediately transferred to sterile nutrient broth for enrichment under aseptic condition and incubated at 37^{0}C for 48 hrs. After 48 hours, loopful of culture from enriched nutrient broth was plated on selective media, crystal violet agar so as to get well isolated colonies. Suspected colonies of *Staphylococci* showing typical cultural characteristics on selective media were picked up and were maintained on nutrient agar slant for further identification.

Identification of Isolates:

Isolates were identified on the basis of morphological, cultural & biochemical characteristics and the results were compared with Bergey's Manual of Determinative Bacteriology 9th edition as well as confirmed by biochemical identification using Vitek 2 System.

Preparation of inoculums:

A loopful of culture from slants was inoculated in into the screw cap tube containing 5ml sterile nutrient broth and incubated at 37^{0}C for 24hrs. Again loopful of culture from same broth was transferred to fresh 5ml of sterile nutrient broth and incubated at 37^{0}C for 6-8 hrs. Turbidity was adjusted according to 0.5 McFarland standards which were then used as an inoculum which corresponds to size of 1.5×10^8 CFU/ml. This suspension was used as inoculums.

Antibiotic Susceptibility Test:

Antimicrobial susceptibility testing was performed by the disc diffusion method with commercially available discs (HiMedia, Mumbai, India) of Rifampicine (RIF) 5 mcg, Amikacin(AK) 30 mcg, Ampicillin (AMP) 10 mcg, Sparfloxacin (SPX) 5mcg, Cefamandole (FAM) 30 mcg, Cefepime(CPM) 30 mcg, Cefonicid(CID) 30 mcg, Cefotaxime(CTX) 30 mcg, Ceftizoxime(CZX) 30 mcg, Cephalothin(CEP) 30 mcg, Erythromycine(E) 15 mcg, Gatifloxacin(GAT) 5 mcg, Gentamicin(GEN) 10 mcg, Linezolid(LZ) 30 mcg, Moxifloxacin(MO) 5 mcg and Oxacillin(OX) 1 mcg. Selected antibiotic discs placed over plates seeded with 100 ml broth culture (0.5 McFarland standards) over surface of Hi sensitivity test agar and plates were kept undisturbed in a refrigerator for 1 hour. Then plates were removed from refrigerator and shifted to incubator maintained at 37^{0}C for 18-24hrs. After incubation all plates were examined for zone of inhibition and Zone of inhibition was noted down. Isolates were considered susceptible, intermediate, or resistant to a particular antimicrobial agent on the basis of the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the performance standard for antimicrobial disk susceptibility test, CLSI (CLSI 2007) (formerly NCCLS)

Collection and maintenance of Plant Material: The spice, Seeds of *T. ammi* (ajwain) were purchased from local market of Nagpur (MS). The seeds were dried under shade, pulverized with hand mortar and pestle, filled in air tight bottle and stored in refrigerator till its use.

Preparation of herb extracts:

Hot extract (Soxhlet extract):

Over 25 g of powdered herb were filled in Soxhlet thimble and refluxed in Soxhlet apparatus with 250 ml of desired solvent for 24 hrs. Solvent were used in a sequence with increasing order of polarity of the solvent, petroleum ether (60^{0}C- 80^{0}C), chloroform, acetone, methanol and water. Before every extraction, material inside the thimble was exposed to air to evaporate preceding solvent absorbed in material. After 24 hrs solvent is recovered till approximately 55 -60ml of extract remained in round bottom flask. The solvents were then evaporated at appropriate temperature for each solvent until a very concentrated extract (less than 50 ml) was obtained and final volume was make up to 50 ml in volumetric flask.

Cold extract:

25 g of powdered herb were tide loosely in nylon cloth (400 mesh) and immersed in 100 ml of solvent in 250 ml stoppered conical flask and kept at 25^{0}C under 150 rpm in rotary B.O.D incubator for 24 hrs. Successive extraction of same powdered material was carried out in selected series of solvents as used in hot extraction. Before every extraction, material was exposed to air to evaporate preceding solvent absorbed in material. After 24 hrs solvent is evaporated at room temperature to obtain final volume of 50 ml.

Total 10 extracts were prepared and they were abbreviated and hereafter referred by their abbreviation. They are Hot Ajwain Acetone extract (HAAT), Cold Ajwain Acetone extract (CAAT), Hot Ajwain Methanol extract (HAME), Cold Ajwain Methanol extract (CAME), Hot Ajwain Chloroform extract (HACF), Cold Ajwain Chloroform extract (CACF), Hot Ajwain Water extract (HAW), Cold Ajwain Water extract (CAW), Hot Ajwain Petroleum ether 60^{0}C- 80^{0}C extract (HAPE) and Cold Ajwain Petroleum ether 40^{0}C- 60^{0}C extract (CAPE).

Cold and hot concentrated extract was prepared in sufficient volume (50 ml) and preserved at 4 C in sealed vials for further use to avoid batch to batch variations.

Sterility testing of Plant extracts:

100 µl of hot and cold extract were spread on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) plates, and checking for growth of bacteria in 48 hrs at 37^{0}C and at 25^{0}C and fungal contaminants after 1 week of incubation at room temperature (Sule LO., 2008) to ensure absence of any microbial contamination.

Testing of Synergistic activity:

100 µl of extract was transferred to sterile petri plates and 15ml of sterilized molten Hi-sensitivity test agar maintained 55^{0}C in constant temperature water bath was then poured in a plate, then plate is rotated for about 30 to 35 seconds to ensure even mixing of extract with the agar medium. Agar medium was then allowed to solidify. 100 µl of inoculum was added on solidified Hi-sensitivity test agar and spread over agar medium with the help of sterile disposable L-spreader. Four or five antibiotic discs were placed

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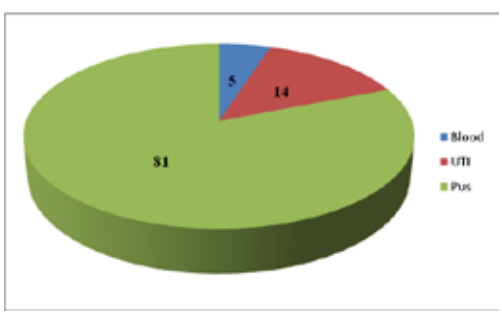
over it equidistantly. Plates were kept undisturbed in a refrigerator for 1 hour. Then plates were removed from refrigerator and shifted to incubator maintained at 37^{0}C for 18-24hrs. After incubation all plates were examined for zone of inhibition and Zone of inhibition was noted down. Zone of inhibition is then measured and classified as susceptible, intermediate, or on the basis of manufacturer's interpretive table, CLSI standard.

Result and discussion:

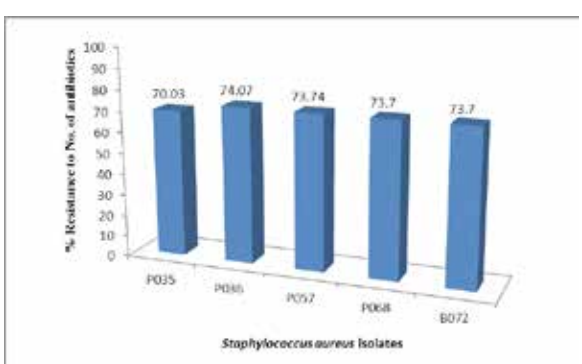
Total 106 Clinical samples of urine, pus, blood and sputum were collected from different pathology laboratories of Nagpur (MS), India for isolation of *Staphylococcus sp.* Total 21 suspected isolates of *Staphylococcus aureus* were identified on the basis of morphological, cultural & biochemical characteristics and all of them were found to be *Staphylococcus aureus.* 17 isolates were associated with pus samples, 3 with urinary tract infection (UTI) and 1 was associated with blood. Percentage prevalence of *Staphylococcus aureus* in clinical samples are graphically illustrated in Graph 1. More than 80% *Staphylococcus aureus* were found associated with pus samples, 14 % and 5 % with Urine samples and blood samples respectively. *Staphylococcus aureus* interpreted to be the most common cause of skin infection and development of MDR against commonly used antibiotics making the case difficult to manage.

Graph 1: Graph showing % prevalence of *Staphylococcus aureus* in clinical samples.



These 21 isolates were preliminary screened for the selection of MDR using 16 different antibiotics. Antibiogram study of these isolates revealed that all these isolates are resistant to several antibiotics out of 16 antibiotics tested. Total 5 MDR isolates of *Staphylococcus aureus* namely P035, P036, P057, B072 and P068 were selected for further studies out of 21 isolates on the basis of their resistance to more than 70% antibiotics. Graph 2 illustrates the % resistance of each isolate to number of antibiotics.

Graph 2: Graph showing resistance of isolate to % number of antibiotics.



These isolates were resistant to Rifampicine, Amikacin, Ampicillin, Sparfloxacin, Cefamandole, Cefonicid, Ceftizoxime, Cephalothin, Erythromycine, Gentamicin, Linezolid, Moxifloxacin, Oxacillin and mostly intermediate to Cefepime. Total 10 extracts of ajwain were studied; HAAT, CAAT, HAME, CAME, HACF, CACF, HAW, CAW, HAPE and CAPE. The results of antibiogram and synergistic effect of herbal extracts on susceptibility of MDR isolates are given in Table 1. All extract found to be potentiate the activity of all antibiotics except Cefamandole (FAM), Cefonicid(CID), Erythromycine(E) and Cefepime(CPM). These extracts found to potentiate the susceptibility of even sensitive antibiotic, Gatifloxacin(GAT). HACF extract exhibited higher synergistic activity than others because of solubility of phytochemicals at that particular polarity, on Rifampicine(RIF), Amikacin(AK), Ampicillin (AMP), Sparfloxacin (SPX), Ceftizoxime(CZX), Cephalothin(CEP), Gentamicin(GEN), Linezolid(LZ), Moxifloxacin(MO) and Oxacillin(OX). Synergistic study of extracts of *T. ammi* seeds and antibiotics on susceptibility of resistant *Staphylococcus* isolates showed that most of the extracts exhibited potentiating effect on antibiotics.

The whatever agents having capacity to kill the microbes or arrest its multiplication, are called the antimicrobial agents and there are plenty of antimicrobial agents of which some are discovered or established and some are still hidden in the nature, many of them are in the plants. Zaika long before has reviewed the antimicrobial effectiveness of spices and herbs (Zaika L.L., 1975). The major phenolic compound present in *T. ammi* is thymol and has been proposed to have a germicidal, antispasmodic, and antifungal activity (Burt S., 2004). The clove oil and cinnamon oil have shown nearly an equal activity but, clove oil has maximum activity against *Staphylococcus* with diameter 30mm as compared to Gentamycin 19mm (Amit kumar *et. Al., 2012*). Microorganisms, despite of being small it causes a very profound damage to human body as well as to other living organisms. When acetone and aqueous extracts of *T. ammi* were tested against strains of *Enterococcus faecalis, Staphylococcus aureus, Salmonella typhi, Salmonella typhimurium and Shigella flexneri* by using agar diffusion assay, an inhibitory effect was observed (Gurinder JK and Daljit SA., 2008) which is in line with the present study. Last few decade witnessed an increase in the investigations on plants as a source of solution over human disease management and a search for more natural antimicrobials agents such as extracts from plants as inhibitory compounds. Methanolic extract of seed of *T. ammi* tested against bacterial species of *Pseudomonas aeruginosa* and *Basillus pumilus*; *Staphylococcus aureus* and *Staphylococcus epidermidis* and showed significant antibacterial activity (Shahidi B., 2004). Several studies have been focused on the application of individual extracts derived from plants and some studies showed single extracts have more antimicrobial activity compared to the mixture of major components (Ibrahim S.A. *et. Al., 2006*). Ethanol and acetone extracts of the cinnamon and *T. ammi* has been studies against *Pseudomonas sp., E. coli, Bacillus subtilis* and *Staphylococcus aureus.* Crude ethanol extracts of cinnamon and ajwain are found to be effective in inhibiting the growth of *Pseudomonas sp., Bacillus subtilis* and *Staphylococcus aureus and E. coli* whereas, crude acetone extract of cinnamon and *T. ammi* showed antimicrobial activities against *Pseudomonas sp., Bacillus subtilis* and *Escherichia coli* and not against *Staphylococcus aureus* (Masih usha *et. al.,* 2012). Biomolecules of plant origin appeared to be one of the alternatives over the control of few MDR pathogens. Researchers across the globe have studied the antimicrobial activities of indigenous herbs and spices. Our study provides insight for exploitation of herbal extractives for potentiating the activity of antibiotics against MDR pathogens.

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Conclusion:

Results in present revealed the combined use of plant extracts and antibiotics is useful in the treatment of infectious diseases, and also able to deal with MDR pathogen, *Staphylococcus aureus* which can solve the problem in treatment of emerging drug resistance associated diseases.

The different solvent extracts of *T. ammi* showed significant activity against MDR pathogens in combination with antibiotics which were previously least effective or ineffective. HACF extract exhibited higher synergistic activity than others because of solubility of phytochemicals at that particular polarity. This clears the view of validity of synergistic activity of plant extract with antibiotic which is a promising approach in the field of medical microbiology and provides a new hope for development of drug therapy. Further studies are needed to confirm the phenomenon of synergy between drugs and plant extracts and to avoid allergy and other side effects.

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Table 1: Antibiogram and synergistic effect of herbal extracts and antibiotics on MDR *Staphylococcus* isolates.

Extracts	Antibiotics															
	RIF	AK	AMP	SPX	FAM	CPM	CID	CTX	CZX	CEP	E	GAT	GEN	LZ	MO	OX
P035																
Antibiotic	11(R)	17 (S)	11 (R)	13 (R)	-(R)	18 S	10 (R)	22 (I)	- (R)	- (R)	12(R)	16(R)	24 (S)	11(R)	18(R)	-(R)
HAAT	20 (S)	21 (S)	14 (I)	28 (S)	15 (I)	14 (R)	10 (R)	14 (R)	18 (I)	21 (S)	10 (R)	36 (S)	25 (S)	34 (S)	31 (S)	12 (R)
CAAT	20 (S)	22 (S)	12 (I)	27 (S)	16 (I)	12 (R)	22 (S)	15 (I)	19 (I)	16 (I)	10 (R)	34 (S)	20 (S)	32 (S)	28 (S)	- (R)
HAME	22 (S)	20 (S)	15 (I)	35 (S)	18 (S)	15 (I)	18 (S)	14 (R)	14 (I)	14 (R)	12 (R)	37 (S)	21 (S)	30 (S)	32 (S)	- (R)
CAME	19 (S)	21 (S)	15 (I)	27 (S)	16 (I)	15 (I)	19 (S)	16 (I)	17 (I)	16 (I)	11 (R)	35 (S)	22 (S)	28 (S)	27 (S)	- (R)
HACF	19 (S)	19 (S)	14 (I)	37 (S)	18 (S)	13 (R)	- (R)	16 (I)	19 (I)	16 (I)	10 (R)	27 (S)	24 (S)	29 (S)	26 (S)	- (R)
CACF	21 (S)	29 (S)	14 (I)	30 (S)	19 (S)	15 (I)	14 (R)	15 (I)	13 (R)	16 (I)	12 (R)	35 (S)	24 (S)	31 (S)	35 (S)	- (R)
HAW	22 (S)	20 (S)	13 (I)	26 (S)	14 (R)	11 (R)	19 (S)	15 (I)	17 (I)	17 (I)	10 (R)	31 (S)	23 (S)	31(S)	28 (S)	- (R)
CAW	22 (S)	23 (S)	14 (I)	27 (S)	16 (I)	11(R)	10 (R)	14 (R)	13 (R)	12 (R)	10 (R)	40 (S)	23 (S)	30 (S)	29 (S)	- (R)
HAPE	20 (S)	26 (S)	- R	36 (S)	16 (I)	16 (I)	10 (R)	17 (I)	14 (R)	19(S)	10 (R)	33 (S)	24 (S)	33 (S)	29 (S)	- (R)
CAPE	25 (S)	27 (S)	14 (I)	40 (S)	16 (I)	14 (I)	12 (R)	21 (I)	16 (I)	26 (S)	10 (R)	35 (S)	29 (S)	33(S)	40 (S)	11 (R)
P036																
Antibiotic	10 (R)	19 (S)	- (R)	13 (R)	- (R)	18 (S)	- (R)	24 (S)	- (R)	- (R)	12 (R)	21 (S)	25 (S)	13 (R)	18 (R)	12 (R)
HAAT	20 (S)	22 (S)	15 (I)	25 (S)	18 (S)	16 (I)	12 (R)	17 (I)	19 (I)	22 (S)	10 (R)	38 (S)	27 (S)	34 (S)	28 (S)	11 (R)
CAAT	21 (S)	24 (S)	14 (I)	28 (S)	15 (I)	14 (R)	22 (S)	16 (I)	20 (S)	16 (R)	10 (R)	35 (S)	22 (S)	32 (S)	30 (S)	-(R)
HAME	21 (S)	19 (S)	17 (I)	33 (S)	19 (S)	16 (I)	19 (S)	17 (I)	15 (I)	17 (I)	12 (R)	37 (S)	22 (S)	31 (S)	30 (S)	-(R)
CAME	21 (S)	23 (S)	15 (I)	29 (S)	13 (R)	17 (I)	19 (S)	18 (I)	19 (I)	18 (S)	11 (R)	36 (S)	24 (S)	28 (S)	27 (S)	-(R)
HACF	25 (S)	27 (S)	15 (I)	38 (S)	19 (S)	15 (I)	10 (R)	18 (I)	20 (S)	18 (S)	10 (R)	25 (S)	26 (S)	30 (S)	28 (S)	-(R)
CACF	22 (S)	25 (S)	18 (I)	32 (S)	21 (S)	17 (I)	16 (I)	16 (I)	14 (R)	18 (S)	11 (R)	38 (S)	27 (S)	32 (S)	38 (S)	-(R)
HAW	25 (S)	22 (S)	15 (I)	28 (S)	16 (I)	13 (R)	22 (S)	18 (I)	19 (I)	18 (S)	10 (R)	33 (S)	21 (S)	32 (S)	20 (S)	-(R)
CAW	20 (S)	25 (S)	16 (I)	25 (S)	19 (S)	12 (R)	13(R)	14 (R)	12 (R)	13 (R)	- (R)	40 (S)	25 (S)	31 (S)	30 (S)	-(R)
HAPE	22 (S)	26 (S)	- (R)	38 (S)	15 (I)	18 (R)	-(R)	19 (I)	18 (I)	15 (I)	10 (R)	34 (S)	28 (S)	32 (S)	30 (S)	-(R)
CAPE	28 (S)	25 (S)	16 (I)	41 (S)	18 (S)	15 (I)	14 (R)	23 (R)	18 (I)	25 (S)	10 (R)	37 (S)	30 (S)	32 (S)	41 (S)	-(R)
P057																
Antibiotics	10 (R)	14 R	11 (R)	13 (R)	11 (R)	17 (I)	10 (R)	23 (S)	-(R)	-(R)	12 (R)	18 (R)	25 (S)	13 (R)	18 (R)	-(R)
HAAT	19 (S)	24 (S)	17 (I)	28 (S)	21 (S)	18 (R)	14 (R)	19 (I)	21 (S)	25 (S)	10 (R)	37 (S)	29 (S)	36 (S)	24 (S)	-(R)
CAAT	24 (S)	22 (S)	16 (I)	30 (S)	18 (S)	16 (I)	24 (S)	19 (I)	22 (S)	18 (S)	- (R)	39 (S)	24 (S)	33 (S)	29 (S)	10 (R)
HAME	18 (S)	21 (S)	19 (I)	34 (S)	22 (S)	18 (R)	21 (S)	16 (I)	17 (I)	19 (S)	12 (R)	38 (S)	21 (S)	31 (S)	30 (S)	-(R)
CAME	19 (S)	21 (S)	18 (I)	31 (S)	14 (R)	16 (I)	21 (S)	19 (I)	17 (I)	19 (S)	11 (R)	37 (S)	22 (S)	31 (S)	30 (S)	-(R)
HACF	24 (S)	28 (S)	17 (I)	37 (S)	20 (S)	16 (I)	- (R)	19 (I)	21 (S)	19 (S)	10 (R)	28 (S)	27 (S)	30 (S)	30 (S)	-(R)
CACF	24 (S)	22 (S)	19 (I)	33 (S)	22 (S)	18 (R)	17 (I)	17 (I)	15 (I)	19 (S)	- (R)	36 (S)	27 (S)	32 (S)	31 (S)	11 (R)
HAW	26 (S)	25 (S)	17 (I)	26 (S)	17 (I)	14 (R)	24 (S)	18 (I)	19 (I)	20 (S)	- (R)	38 (S)	22 (S)	32 (S)	30 (S)	- (R)
CAW	19 (S)	24 (S)	18 (I)	24 (S)	18 (S)	12 (R)	13 (R)	15 (I)	18 (I)	17 (I)	10 (R)	34 (S)	29 (S)	31 (S)	30 (S)	- (R)
HAPE	28 (S)	25 (S)	16 (I)	41 (S)	18 (S)	17 (I)	16 (I)	24 (S)	20 (S)	25 (S)	-(R)	38 (S)	30 (S)	32 (S)	41 (S)	- (R)
CAPE	28 (S)	25 (S)	-(R)	38 (S)	16 (I)	18 (R)	16 (I)	17 (I)	18 (I)	25 (S)	-(R)	37 (S)	30 (S)	31 (S)	41 (S)	- (R)
B072																
Antibiotics	-(R)	12 (R)	-(R)	13 (R)	-(R)	17 (I)	10 (R)	22 (S)	-(R)	11 (R)	11 (R)	20 (I)	23 (S)	13 (R)	17 (R)	-(R)
HAAT	22 (S)	21 (S)	15 (I)	27 (S)	14 (R)	15 (I)	-(R)	15 (I)	18 (I)	22 (S)	11 (R)	35 (S)	14 (I)	34 (S)	30 (S)	12 (R)
CAAT	26 (S)	19 (S)	17 (S)	28 (S)	13 (R)	14 (R)	-(R)	16 (I)	19 (I)	21 (S)	12 (R)	36 (S)	16 (S)	37 (S)	31 (S)	-(R)
HAME	27 (S)	21 (S)	18 (S)	36 (S)	15 (I)	15 (I)	-(R)	17 (I)	20 (S)	19 (S)	-(R)	32 (S)	18 (S)	36 (S)	30 (S)	11 (R)
CAME	21 (S)	21 (S)	22 (S)	29 (S)	14 (R)	16 (R)	11 (R)	15 (I)	21 (S)	18 (S)	12 (R)	36 (S)	20 (S)	32 (S)	30 (S)	-(R)

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HACF	26 (S)	24 (S)	28 (S)	34 (S)	13 (R)	14 (R)	-(R)	18 (I)	29 (S)	26 (S)	11 (R)	38 (S)	14 (I)	36 (S)	32 (S)	-(R)
CACF	22 (S)	22 (S)	21 (S)	29 (S)	12 (R)	15 (I)	-(R)	17 (I)	23 (S)	21 (S)	11 (R)	37 (S)	18 (S)	32 (S)	27 (S)	-(R)
HAW	22 (S)	18 (S)	17 (S)	27(S)	11 (R)	17 (I)	-(R)	16 (I)	21 (S)	19 (S)	10 (R)	35 (S)	17 (S)	33 (S)	26 (S)	-(R)
CAW	21 (S)	21 (S)	18 (S)	26 (S)	12 (R)	18 (R)	10 (R)	15 (I)	21 (S)	19 (S)	12 (R)	32 (S)	16 (S)	32 (S)	29 (S)	-(R)
HAPE	24 (S)	17 (S)	20 (S)	28 (S)	14 (R)	17 (I)	-(R)	14 (R)	23 (S)	21 (S)	11 (R)	31 (S)	20 (S)	29 (S)	27 (S)	-(R)
CAPE	24 (S)	21 (S)	21 (S)	27 (S)	13 (R)	16 (I)	-(R)	17 (I)	24 (S)	22 (S)	10 (R)	30 (S)	21 (S)	28 (S)	27 (S)	11 (R)
P068																
Antibiot-ics	-(R)	12 (R)	-(R)	13 (R)	10 (R)	17 (I)	10 (R)	23 (S)	-(R)	-(R)	12 (R)	21 (I)	25 (S)	13 (R)	16 (R)	10 (R)
HAAT	21 (S)	21 (S)	21 (S)	27 (S)	13 (R)	16 (I)	(R)	17 (I)	24 (S)	22 (S)	10 (R)	30 (S)	22 (S)	28 (S)	27 (S)	11 (R)
CAAT	22 (S)	17 (S)	20 (S)	28 (S)	14 (R)	17 (I)	(R)	14 (R)	23 (S)	21 (S)	11 (R)	31 (S)	24 (S)	29 (S)	27 (S)	(R)
HAME	21 (S)	21 (S)	18 (S)	26 (S)	12 (R)	18 (I)	10 (R)	15 (I)	21 (S)	19 (S)	12 (R)	32 (S)	25 (S)	32 (S)	29 (S)	-(R)
CAME	22 (S)	18 (S)	17 (S)	27 (S)	11 (R)	17 (I)	-(R)	16 (I)	21 (S)	19 (S)	10 (R)	35 (S)	19 (S)	33 (S)	26 (S)	-(R)
HACF	22 (S)	22 (S)	21 (S)	29 (S)	12 (R)	15 (I)	-(R)	17 (I)	23 (S)	21 (S)	11 (R)	37 (S)	18 (S)	32 (S)	27 (S)	-(R)
CACF	21 (S)	21 (S)	23 (S)	31 (S)	13 (R)	14 (R)	-(R)	18 (I)	22 (S)	17 (S)	11 (R)	38 (S)	17 (S)	31 (S)	28 (S)	10 (R)
HAW	21 (S)	21 (S)	22 (S)	29 (S)	14 (R)	16 (I)	10 (R)	15 (I)	21 (S)	18 (S)	12 (R)	36 (S)	16 (S)	32 (S)	30 (S)	-(R)
CAW	24 (S)	21 (S)	18 (S)	36 (S)	15 (I)	15 (I)	-(R)	17 (I)	20 (S)	19 (S)	-(R)	32 (S)	22 (S)	36 (S)	30 (S)	11 (R)
HAPE	22 (S)	19 (S)	17 (S)	28 (S)	13 (R)	14 (R)	-(R)	16 (I)	19 (I)	21 (S)	12 (R)	38 (S)	21 (S)	37 (S)	31 (S)	-(R)
CAPE	26 (S)	21 (S)	15 (I)	27 (S)	14 (R)	15 (I)	10 (R)	15 (I)	18 (I)	22 (S)	11 (R)	35 (S)	25 (S)	34 (S)	30 (S)	10 (R)

(S)- Sensitive, (I)- Intermediate, (R)- Resistant

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