Asian J. Dairy & Food Res, 36(2) 2017 : 100-105
Print ISSN:0971-4456 / Online ISSN:0976-0563

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
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A study on the antimicrobial resistant patterns of P*seudomonas Aeruginosa* isolated from raw milk samples in and around Tirupati, Andhra Pradesh.

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Received: 19-12-2016 Accepted: 08-04-2017 DOI: 10.18805/ajdfr.v36i02.7951

ABSTRACT:
This study was aimed to detect the prevalence of *Pseudomonas aeruginosa* in milk (n=125) samples which were collected from local vendors, private dairy farms in and around Tirupati. Pre-enrichment was done by taking 10ml of each sample and inoculated in 90 ml of Tryptone Soya broth and incubated at 37^{0}C for 24hrs. A loopful of culture was taken from broth and streaked on nutrient agar and Cetrimide agar plates and incubated at 37^{0}C for 24hrs which were further confirmed by biochemical tests. The nineteen positive samples for *P.aeruginosa* were further tested for antimicrobial susceptibility which has shown multi drug resistant ranging from four to twelve antimicrobials and Multiple Antibiotic Resistance (MAR) index ranges from 0.33 to 1.The isolates of *P.aeruginosa*in the present study are highlyresistant to ampicillin, penicillin, and oxacillin (100%) and maximum sensitive to Vancomycin (5.3%) followed by Tetracycline (10.5%).

Key words: Antimicrobial Resistance, Milk, Multiple Antibiotic Resistance index (MAR index), *Pseudomonas aeruginosa.*

INTRODUCTION

The control of microbial spoilage of livestock products is crucial for the quality and safety of the foods(Zhang *et al.,* 2014) which requires an understanding of a number of factors including the knowledge of possible hazards, their likely occurrence in different products, their physiological properties and the availability and effectiveness of different preventive measures(Blackburn, 2006). Among the foods of animal origin, milk is a significant food of human nutrition owing to its high nutritional value. It is naturally a good medium for the growth of microorganisms. Quality control of milk and milk products are therefore of paramount importance with a view to reduce food poisoning outbreaks (Bashir *et al.,* 2014).

Among the spoilage bacteria, psychotropic bacteria have become an escalating problem for the food industry after introduction of refrigerated storage of raw and processed foods. The main psychotropic bacteria present in raw milk are Gram negative rods i.e. *Pseudomonas* species, comprising at least 50 percent of the total bacteria in refrigerated foods. *Pseudomonas* species, particularly *P.aeruginosa* have been the critical cause in majority of outbreaks of inflammatory infections. It is also responsible for a variety of systemic infections like urinary tract infections, respiratory and gastrointestinal tract infections, dermatitis, bacteraemia, soft tissue infections, bone and joint infections etc. Due to its nominal nutritional requirement *P.aeruginosa* has the ability to survive in soil, plant surface, waste water, moist environment, surface waters or even on inert materials.

P.aeruginosa is common in the farm environment particularly in water supplies and contaminated water used for udder washing, and often been cited as a source of infection for pseudomonas mastitis in cows. This organism is highly ubiquitous in water system and capable of acquiring antibiotic resistance due to its low outer membrane permeability and extensive efflux pump system and is resistant to various antibiotics; therefore it is particularly dangerous and dreaded pathogen.

P.aeruginosa infection may cause economic loss due to its ability to reduce the quality of hide. On the other hand this bacterium may contaminate milk which is the predisposing organism of mastitis and may cause infection in immuno-compromised individuals, transmitted through consumption of contaminated milk. However very little work was carried out on the isolation of *P.aeruginosa* from milk samples and the data is not uniform. Keeping in view of the severity of the infections caused by *P.aeruginosa* among the animal population and its public health significance this study was carried out to isolate *P.aeruginosa* from milk and to determine the antibiotic resistant patterns of the isolates to some commonly used antibiotics.

MATERIALS AND METHODS

Sample collection: A total of 125 milk samples were collected for isolation and identification of *Pseudomonas aeruginosa* from organized dairy farms, unorganized dairy farms and from local milk vendors in and around Tirupati, Andhra Pradesh, India. Milk sample of 25 ml was collected aseptically from each animal in a sterile screw capped bottle which was autoclaved previously. The samples were

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Table 1. Antibiogram of *Pseudomonas aeruginosa* isolated from milk samples

Antibiotics used	n=19					
	Resistant		Intermediate resistant		Sensitive	
	no. of isolates	%	no. of isolates	%	no. of isolates	%
Tetracycline	15	78.94	2	10.5	2	10.5
Erythromycin	10	52.63	2	10.5	7	36.8
Co-Trimaxozole	15	78.94	1	5.3	3	15.8
Ampicillin	19	100	0	0.0	0	0.0
Ciprofloxacin	7	36.84	2	10.5	10	52.6
Penicillin	19	100	0	0.0	0	0.0
Chloramphenicol	11	57.89	2	10.5	6	31.6
Azithromycin	10	52.63	3	15.8	6	31.6
Streptomycin	9	47.36	6	31.6	4	21.1
Gentamicin	8	42.10	2	10.5	9	47.4
Vancomycin	17	89.47	1	5.3	1	5.3
Oxacillin	19	100	0	0.0	0	0.0

collected in sterile conditions by discarding first few ml of milk from all quarters of udder during milking. In case of local vendors, milk was collected directly from their vessels by using a sterile dipper and the same was transferred to screw capped vials. Immediately after collection, all the samples were kept in ice box in which temperature was maintained at $4^0c \pm 1^0c$ and transported to the laboratory of Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati for further processing.

Isolation and Identification of *P.aeruginosa:* 10 ml of each milk sample was inoculated into 90 ml of Tryptone Soya Broth (Himedia Pvt. Ltd, India) and incubated at 37^0C for 24 hrs. After incubation, a loopful of culture was taken and streaked on both nutrient agar (Himedia Pvt. Ltd, India)and Cetrimide agar (Himedia Pvt. Ltd, India) simultaneously. These plates were incubated at 37^0C for 24 hrs. Cetrimide agar is a selective and differential medium for the identification of *Pseudomonas aeruginosa* in which cetrimide acts as detergent which inhibits most bacteria and enhances the production of two pigments pyocyanin and pyoverdin.

Biochemical characterization: The suspected colonies on cetrimide agar were collected and screened for grams staining and biochemical characterization which includes Indole test, methyl red test, voges proskauer test, citrate utilization test, Triple Sugar Iron test and catalase test. All these tests were performed according to the protocols mentioned in Medical Microbiology (Robert Cruikshank, 1975).

Determination of antibiotic susceptibility test (ABST) by Disc Diffusion method: *Pseudomonas aeruginosa* isolates were tested for antibiotic susceptibility by the Kirby-Bauer disc diffusion method on Mueller Hinton agar using commercial discs (Himedia Pvt. Ltd, India). Initially the test was performed by inoculating the test colonies in nutrient broth and incubated at 37^0C for 24 hrs. Later, Muller Hinton agar plates were swabbed with nutrient broth culture with the help of sterile swab and antibiotic discs were placed carefully on swabbed surface of plates. These plates were incubated at 37^0 C for 24 hrs and zones of inhibition for different antibiotics were measured. The antibiotics used in this test and the antibiogram of P.aeruginosa were given in the Table 1.

MAR index: The multiple antibiotic resistance index (MAR index) was determined for each isolate by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotics tested (Piyush Tripathy *et al.,* 2011) and given in the Table 2.

$$\text{MAR index} = \frac{\text{Number of antibiotics to which isolate is resistant}}{\text{Total number of antibiotics used in this test}}$$

Table 2: MAR index of *Pseudomonas aeruginosa* isolated from raw milk samples in and around Tirupati of Chittoor in Andhra Pradesh

Isolate No.	MAR Index	Antibiotics
1	0.67	TE, E, COT, Amp,P,C, VA, Ox
2	1	TE, E, COT, AMP, CIP, P, C, AT, S, GEN, VA, OX
3	0.67	E, COT, AMP, P, AT, S, VA, OX
4	1	TE, E, COT, AMP, CIP, P, C, AT, S, GEN, VA, OX
5	1	TE, E, COT, AMP, CIP, P, C, AT, S, GEN, VA, OX
6	0.67	E, COT,AMP, P, C, AT,VA, OX
7	0.58	TE, COT, AMP,P, C, VA,OX
8	0.67	VA,OX,AT, C,P,AMP,COT,TE
9	0.33	OX,P,AMP,COT
10	0.58	OX,VA,C,P,AMP,COT,TE
11	0.58	TE, VA, OX,S, P, AMP, COT
12	0.83	TE,E,COT,AMP,CIP,P, S,GEN, VA,OX
13	0.58	TE, COT, AMP,P,C,VA, OX
14	0.67	TE,E,AMP, P, C, AT, VA, OX
15	0.42	AMP, P, GEN, VA, OX
16	0.75	TE,E, AMP, CIP,P , S, GEN, VA, OX
17	0.92	TE, E, AMP, CIP, P, C, AT, S, GEN, VA, OX
18	0.75	TE, COT,AMP, P, AT, S, GEN, VA, OX
19	0.58	TE,COT, AMP, CIP, P, AT, OX

RESULTS AND DISCUSSION

A total of 19 isolates of *P.aeruginosa* were recovered from 125 milk samples during the study period and confirmed by biochemical tests like Indole test, methyl red test, voges proskauer test, citrate utilization test, Triple Sugar Iron test and catalase test and *P.aeruginosa* isolated from milk in the present investigation is 15.2%. Almost similar incidence rate of *P.aeruginosa* (15.62%) from urinary tract infections was reported in studies of Durgesh *et al.*(2012). Garba *et al.*(2012) reported lower incidence rate (11%) of *P.aeruginosa* from wounds of patients whereas Gad *et al.*(2007) reported 18.2% incidence rate of *P.aeruginosa* from clinical and environmental samples. Keskin and Ekmekci (2007) reported that 21% of the isolates from milk were *P.aeruginosa* which indicates a high percentage of *P.aeruginosa* from milk when compared to the present investigation. In the present investigation the samples were collected from the retail milk vendors who have kept the milk at refrigeration and also from the farm directly without any refrigeration. The presence of *P.aeruginosa* from the milk samples may be due to unhygienic maintenance of the refrigerators or bulk coolers and may be due to contamination of milk with polluted water at farm level.

The Muller Hinton Agar-based antibiogram-resistogram pattern study of *P.aeruginosa* isolated from milk is shown in Table 2 and represented dagrammatically in Figure-1. All the nineteen *P.aeruginosa* strains isolated in this study have shown 100% resistance to ampicillin, penicillin and oxacillin. Antidrug profile of P.aeroginosa was represented in Table-3. Similar to the present investigations, Jombo *et al.*(2008), Tamil *et al.*(2011), Gonuigur *et al.*(2003). Okon *et al.*(2009), Gedamu *et al.*(2014) and Hajira *et al.*(2015) have reported 100% resistance to ampicillin by the isolate of *P.aeruginosa;* on the contrary only 45.4% of the resistance to ampicillin was shown by the isolates of Garba *et al.*(2012). Along with ampicillin,

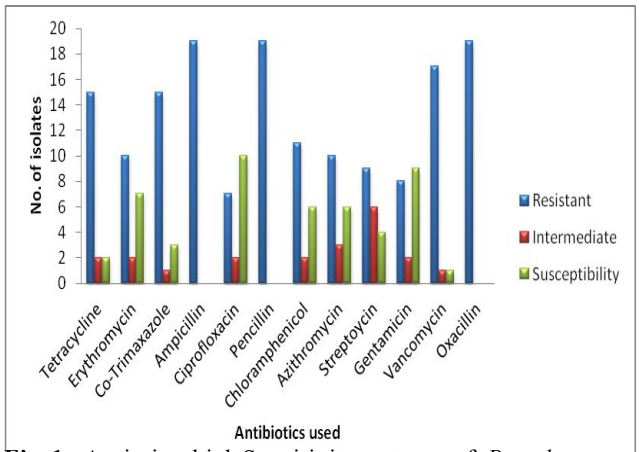

Fig-1: Antimicrobial Sensitivity pattern of *Pseudomonas aeruginosa* isolated from milk samples

penicillin was also shown 100% resistance to the isolates of *P.aeruginosa* in the present investigation. These results are in accordance with the results of Jombo *et al.*(2008) and Gonuigur *et al.*(2003) who also reported 100% resistance to penicillin by the isolates of *P.aeruginosa.* Oxacillin has shown 100% resistance of *P.aeruginosa* in this study. T h e isolates of *P.aeruginosa* in the present investigation have shown 78.9% resistance to tetracycline. Similar to these results Salimi (2009), Gedamu *et al.*(2014), Garba *et al.*(2012) and Tamil *et al.*(2011) have reported 78.3, 75, 81 and 83.3% resistance to tetracyclines by their isolates of *P.aeruginosa* respectively. Whereas 100% resistance to penicillin was reported by Jombo *et al.*(2008) and Hajira *et al.*(2015) where as very little percentage (10%) of resistance to tetracyclines by *P.aeruginosa* isolates was reported by Ogundipeju and Nwobu (2004).

For erythromycin, the isolates of *P.aeruginosa* in the present study have shown 52.63% resistance. A higher resistance than in the present investigation was reported by

Table 3: Anti drug profile of *Pseudomonas aeruginosa* isolated from raw milk samples

antibiotic resistant profile	No. of isolates n=19	Total No. (%) of isolates	Antibiotics that are resistant
Four	1	5.26	OX,P,AMP,COT
Five	1	5.26	AMP, P, GEN, VA, OX
Seven	5	26.32	TE,COT, AMP, CIP, P, AT, OX
			TE, COT, AMP,P,C,VA, OX (3)--------
			TE, VA, OX,S, P, AMP, COT
Eight	5	26.32	TE, E, COT, AMP,P,C, VA, Ox
			E, COT, AMP, P, AT, S, VA, OX
			E, COT,AMP, P, C, AT,VA, OX
			TE,COT,AMP, P, C,AT, OX, VA
			TE,E,AMP, P, C, AT, VA, OX
Nine	2	10.53	TE, COT,AMP, P, AT, S, GEN, VA, OX
			TE,E, AMP, CIP,P , S, GEN, VA, OX
Ten	1	5.26	TE,E,COT,AMP,CIP,P, S,GEN, VA,OX
Eleven	1	5.26	TE, E, AMP, CIP, P, C, AT, S, GEN, VA, OX
Twelve	3	15.79	TE, E, COT, AMP, CIP, P, C, AT, S, GEN, VA, OX

Tamil *et al.*(2011) and Okon *et al.*(2009) who reported 100% resistance to erythromycin by their isolates. Further Emmanuel *et al.*(2012) reported a little higher resistance (90%) to erythromycin than in the present investigation.

Co-trimaxozole was another antibiotic used in this study. The isolates of *P.aeruginosa* have shown 78.94% of resistant to the antibiotic. A little lesser resistance (72.4%) than in the present investigation was reported by Chander *et al.*(2013) where as Gedamu *et al.*(2014) and Tamil *et al.*(2011) have reported 55.5% and 66.6% resistance respectively for co-trimaxazole . Higher resistance to co-trimaxozole by the *P.aerugionsa* isolates was observed by Nwankwo *et al.*(2010), Hajira *et al.*(2015) and Garba *et al.*(2012) who reported 100%, 99% and 90.9% respectively.

In the present study, ciprofloxacin has shown 36.84% of resistance by the isolates of *P.aeruginosa.* Similar to the results obtained in this study,Piyush *et al,* (2011) and Ramana and Chaudhury (2012)have reported 31.37 and 39% resistance to ciprofloxacin by their isolates. A little higher resistance to ciprofloxacin than the isolates of present study was reported by Ahmed *et al.*(2013), Rajat *et al.*(2012), Chander *et al.*(2013)and they reported 40.5%, 49% and 51.72% respectively. *P.aeruginosa* has showna bit more resistance to ciprofloxacin in the works carried out by Al-kabsi *et al.*(2011), Hajira *et al.*(2015), Anjum and Mir (2009), Gad *et al.* (2007) , Zaheer *et al.* (2012) and Ahmed *et al.* (2013) who reported 92, 73, 73, 62, 60 and 56% respectively.

Chloramphenicol has shown 57.89 percent resistance to *P.aeruginosa* isolated in this study. Similar to the results of present investigation Gedamu *et al.* (2014)reported 63.9% of resistance by their isolates where asOkon *et al.* (2009), Ahmed *et al.* (2013)have reported 98.1 % and 73.7% resistance to chloramphenicol by the isolates of *P.aeruginosa*.On the contrary,Gad *et al.* (2007)reported 100% resistance to chloramphenicol by the isolates of *P.aeruginosa.*

In the present investigation Azithromycin has shown 52.63% of resistance by the *P.aeruginosa* isolates. Higher resistance (87%) to Azithromycin by *P.aeruginosa* was reported by Gad *et al.*(2007)than the resistance shown by the isolates of *P.aeruginosa* in this study.

Gentamicin has shown 42.10% resistance to the isolates of *P.aeruginosa* in the present investigation. Similar to the results obtained in the study, Ahmed *et al.*(2013), Garba *et al.*(2012) and Ramana and Chaudhury (2012) have reported 43.9%, 45.4% and 40% resistant to *P.aeruginosa* respectively. A little lesser resistant than resistance observed in the present investigation was reported by Piyush Tripathy *et al.*(2011) and Hajira *et al.*(2015)who reported 29% and 34% of resistance to gentamicin by their isolates respectively. A little higher resistance to gentamicin than in the present study was observed by *P.aeruginosa* isolates in the works carried out by Rajat *et al.*(2012), Tamil *et al.*(2011) and Gad *et al.*(2007) and they reported 63%, 66.6% and 85% of resistance to gentamicin by *P.aeruginosa* isolates respectively, where as okon *et al.*(2009) reported 100% resistance to gentamicin by the isolates of *P.aeruginosa.*

In the present investigation, ampicillin, penicillin and oxacillin have shown 100 percent resistance by *P.aeruginosa* isolates from milk. This high resistance may be due to the fact that the strains isolated from milk samples have been subjected to the selective action of both antibiotics and disinfectants. The isolates have shown varied resistance to the ampicillin, penicillin and oxacillin antibiotics used in the study and the isolates have shown resistance to Vancomycin (89.74%), tetracycline (78.94%), Co-trimaxozole (78.94%), chloramphenicol (57.89%), erythromycin (52.63%) and Azithromycin has shown 52.63% resistance for *P.aeruginosa* isolates in this study. These results are indicating that there is an emergence of multidrug resistant *P.aeruginosa.*

All the isolates of *P.aeruginosa* have shown multi drug resistant ranging from four to twelve antibiotics which is almost similar to studies of Emmanuel *et al,* 2012 who reported multi drug resistant isolates of *P.aeruginosa* ranging from five to eleven antibiotics. While Lateef (2004) reported MAR *Pseudomonas* strains with resistance patterns varying between two to seven antibiotics. All the nineteen isolates of *Pseudomonas aeruginosa* in the present study have shown different MAR indices ranging from 0.33 to 1. Because of mutations in outer membrane porins resulting in reduced permeability to antimicrobials and also due to over expression of multi drug efflux pumps, which tends to pump out antibiotics before they have opportunity to act on their target results in bacterial resistance to multi drug antibiotics (Emmanuel *et al*, 2012).

In view of the current rate of antimicrobial resistance of *P.aeruginosa* as found in the present study, antimicrobial susceptibility testing by microbiology laboratories of hospitals and clinics should be made a routine practice. Also the procurement of reagents and materials for susceptibility testing along with the requisite personnel should be considered as basic laboratory requirement in order to boost this conventional practice.

Effective management of MDR *P.aeruginosa* in the milk by veterinarians would require good background knowledge of the prevailing antimicrobial susceptibility patterns of the organism. Such information would be even more valuable in human health care medicine of the country where reports about the occurrence of antimicrobial resistant *P.aeruginosa* are increasing day by day especially with urinary tract infections.

Regional referral health centres for both veterinary and human medicine should assist in compiling periodic

antimicrobial susceptibility patterns of bacterial isolates. Such data could be made available to the veterinary dispensaries and primary health centres which are not having the facilities to carryout sensitivity tests, so as to help and guide the relevant health personnel on the most suitable antibiotic to prescribe.

CONCLUSION

In conclusion, this study has shown that the rate of antibiotic resistance against *P.aeruginosa* is medium at Tirupati. Prudent and Justifiable reasons for antibiotics consumption both for prophylactic and therapeutic use against mastitis and other infections should be critically evaluated against the effects of antimicrobial resistance. Further antimicrobial susceptibility testing for the isolates should be performed as a basic laboratory procedure among veterinary hospitals so as to find out the suitable antibiotic for the prescription. Antimicrobial sensitivity patterns of the isolates for different antibiotics should be generated by an appropriate referral veterinary hospital and the same should be distributed to the neighbouring veterinary hospitals for regular reference. Finally Vancomycin, Tetracycline followed by Co-Trimaxozole should be recommended on isolation of *P.aeruginosa* in the absence of a viable susceptibility report.

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