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RESEARCH ARTICLE

PREVALENCE AND ANTIBIOGRAM OF YERSINIA ENTEROCOLITICA IN MILK AND FECAL SAMPLES OF DAIRY COWS FROM DIFFERENT PLACES OF TIRUPATHI REGION ANDHRA PRADESH, INDIA

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ABSTRACT

The purpose of this study was to determine the prevalence and antibiogram profile of *Yersinia enterocolitica* and to assess the carrier status for *Y. enterocolitica* in milking cows of Tirupathi region, Andhra Pradesh, South India. Altogether 120 samples were processed for isolation of *Y. enterocolitica* in which 50 samples were raw milk and 50 samples were faecal samples of lactating dairy cows. The samples were collected from different farms located in Tirupathi region, under aseptic conditions. All the samples were processed for isolation and identification of *Y. enterocolitica* as per standard protocol. Evaluation of antibiotic sensitivity pattern of *Y. enterocolitica* was assessed by Kirby-Bauer method. The overall prevalence of *Y. enterocolitica* was observed in 25% of the collected samples which comprising of cow milk (10%) and lactating dairy cow fecal samples (35.71%) based on colony characters and biochemical reactions. All the *Y. enterocolitica* isolates were resistant to more than one antibiotic and no isolates were susceptible to all the antibiotics. *Y. enterocolitica* isolates were highly resistant to Streptomycin and Penicillin (96.67%). Least resistant against Amoxycillin (6.67%) and none was resistant to Co-trimaxazole. The predominant antimicrobial resistance pattern were Streptomycin – Penicillin - Oxacillin with 66.67% of isolates shown multiple antibiotic resistance indices of more than 0.2.

INTRODUCTION

Yersinia enterocolitica is an important food borne zoonotic pathogen known to cause gastrointestinal problems with symptoms ranging from acute enteritis with fever to occasionally bloody watery diarrhea, particularly in children (Tadesse *et al*, 2013).Food borne diseases are foremost international health problems causes the majority of illnesses particularly in developing countries. Among the various food borne illness, yersiniosis is listed in third place after campylobacteriosis and salmonellosis (Zadernowsks *et al*, 2014).

Pigs are assumed to be the main reservoir of pathogenic *Y. enterocolitica* because pig is so far the only animal species from which pathogenic strains have frequently been isolated (Ahomaa *et al*, 2007). Several domestic animals likes dogs, cats, cows, sheep and horses and several wild animals like rodents (mainly mice), monkeys, deer and foxes have also been incriminated as potential reservoirs (Ahomaa *et al*, 2006). Following excretion from the body, these bacteria may survive for a long time in the environment due to their low nutritional requirements and relatively high resistance to unfavourable conditions (Zadernowsks *et al*, 2014).

Y. enterocolitica gastroenteritis cases are sporadic or occur in small clusters, but large outbreaks have reported worldwide in families, schools, hospitals and in association with community gathering (Leclercq *et al*, 2005) although *Y. enterocolitica* has been isolated from a number of environmental, food and water sources, there have been relatively few documented outbreaks of human illness where food was proved by culture to be the source of infection. *Y. enterocolitica* is one of the few human pathogens that can grow at refrigeration temperature and its presence in food is of great public health concern. Foods with animal origins have the higher risk of gastrointestinal disease caused by *Y. enterocolitica* in human. Milk and dairy products

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are the most consumed foods with animal origins. In addition, several studies have reported the presence of *Y. enterocolitica* in milk and dairy products (Rahimi *et al*, 2013). According to (Ackers *et al*, 2000) the three well documented outbreaks in which contaminated chocolate milk, raw milk and tofu were the vehicles of transmission. The psychrotropic nature of this microorganism plays a significant role in occurrence of yersiniosis in humans by consuming refrigerated milk and milk products (Kushal and Anand, 2006).

Antibiotics are commonly used to treat cattle disease especially for mastitis in dairy cows and their indiscriminate use lead to the development of multi- drug resistant strains of bacteria thereby rendering antibiotic treatment ineffective (Sadek *et al*, 2014). Antibiotic therapy for treatment of yersiniosis in humans was not indicated except in systemic and extraintestinal infection and enterocolitis in immuno-compromised patients (Mayrhofer *et al*, 2004). The presence of antimicrobial resistance leads to treatment failures and there is the need for expensive and/ or toxic alternative drugs which in most cases are more expensive (WHO, 2007). The spread of drug resistance among *Y. enterocolitica* is also of concern for public health appraisal. The World Health Organization (WHO) report on infectious diseases in 2000 declared that antibiotic resistance poses a severe threat to human health, and that the problem is growing globally (Pandove *et al*, 2012). There was very few published work on *Yersinia enterocolitica* milk borne infection in South India and there is no previously published report on antibiotic resistant *Y. enterocolitica* isolation from Tirupathi region. Keeping all this in view the present study was aimed to determine the prevalence of the *Yersinia enterocolitica* from cow milk and dairy cow fecal samples collected from Tirupathi region, Andhra Pradesh, South India by conventional culture method and to study its antibiogram pattern with special reference to public health significance.

MATERIALS AND METHODS

Samples collection

A total of 120 samples were collected for isolation of *Yersinia enterocolitica*, which comprising of 50 cow milk samples and 70 dairy cow fecal swabs collected from organized government and private farms of in and around Tirupathi region, South India. Each milk samples (50 – 100 mL) was collected from apparently healthy udder in a sterile screw cap bottle aseptically from all four quarters after discarding the initial 1- 2 mL of milk during milking. The fecal swab was collected from each apparently healthy individual animal using a sterile swab and then inserted into sterile PBS tubes under aseptic conditions. All the samples were maintained on ice, transported to the laboratory of Department of Veterinary Public Health and Epidemiology, College of Veterinary science, Tirupathi and processed within 2 hrs of collection.

Isolation and Identification of Yersinia enterocolitica

For the isolation of *Yersinia enterocolitica*, 10 mL of milk sample and fecal swab was aseptically transferred to 90 mL of Tryptone Soya Broth (Himedia Pvt. Ltd, India) and incubated at 25˚C for 2 days. After incubation, a loopful of culture was streaked on Yersinia Selective agar (YSA) plates which containing Yersinia selective supplement (Himedia Pvt. Ltd, India). The YSA plates were incubated at 25˚C for 48 hrs. Typical dark red colonies resembling bull eye, which are surrounded by a transparent border were considered as presumptive *Y. enterocolitica*.

Biochemical Characterization of Yersinia enterocolitica

The plates shown colonies with suspected morphologies were selected and tested for Gram's method of staining and biochemical characterization which includes oxidase, catalase, utilization of simmon's citrate, methyl red test, voges proskauer test, indole test and triple sugar iron test. All these tests were performed as per standard protocols.

Antibiogram study

Yersinia enterocolitica isolates were tested for antibiotic susceptibility by the Kirby- Bauer disc diffusion method on Mueller Hinton agar using commercial discs (Himedia Pvt. Ltd, India). The following antibiotics were used: Cotrimoxazol (COT, 25µg), Ciprofloxacin (CIP, 5µg), Amoxycillin (AM, 10µg), Gentamicin (GEN, 10µg), Erythromycin (E, 15µg), Vancomycin (VA, 30µg), Enrofloxacin (EX, 10µg), Streptomycin (S, 10µg), Penicillin (P, 10µg), Ampicillin (AMP, 10µg), Chloramphenicol (C, 30µg), Oxacillin (OX, 1µg), Azithromycin (AZM, 15µg) and Tetracyclin (TE, 30µg). Antibiotic susceptibility to all these 14 antibiotics were performed for 30 *Yersinia enterocolitica* isolates according to the criteria of the National Committee for Clinical Laboratory Standards. Diameters of the zone of inhibition around the disc were measured manually by using measuring scale to the nearest millimeter using standard chart and the isolates were classified as sensitive, intermediate and resistant according to the National Committee for Clinical Laboratory Standards (Wayne, 2002).

RESULTS AND DISCUSSION

Prevalence of Yersinia enterocolitica

Yersinia enterocolitica has been isolated from animals (Okwori *et al*, 2005), raw food materials, environment (Fredriksson-Ahomaa and Korkeaka, 2003), water and human beings (Okwori *et al*, 2007). In our study we chosen the milking cows to determine the carrier status for *Y. enterocolitica* by tested their milk and fecal samples.

A total of 120 samples comprising of 50 raw cow milk samples and 70 dairy cow fecal samples were processed for isolation and identification of *Yersinia enterocolitica* by conventional culture and biochemical characterization. *Y. enterocolitica* isolates were obtained from enriched samples by selective plating on YSA. Typical dark pink centers surrounded by translucent border (Fig 1) were selected for gram staining (Fig 2) and biochemical characterization (Table 1, Fig 3 and 4) for further confirmation.

Table 1 Biochemical Characterization of E.coli

Biochemical test	Reaction
Indole test	Positive
Methyl Red	Positive
Voges- Proskauer	Positive
Citrate utilization	Negative
Urease Production	Positive
H_2S production	Negative
Oxidase	Negative
Catalase	Positive
Triple sugar Iron agar	Acid butt (Yellow), Alkaline Slant (Yellow) without gas production

Yersinia enterocolitica colony in Yersinia Selective agar

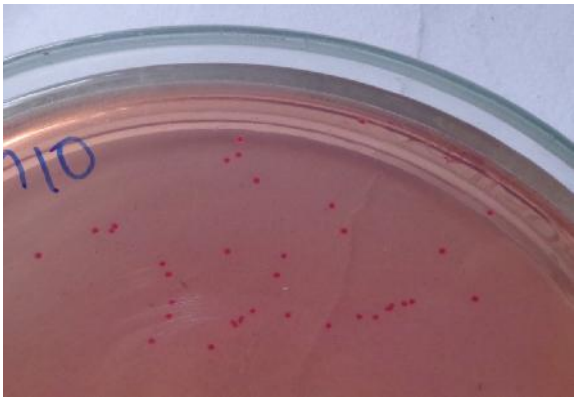

Figure 1. Culture plate showing growth of dark pink centre colony surrounded by translucent border (bull eye appearance) in Yersinia Selective agar.

Gram staining for *Yersinia enterocolitica* colony

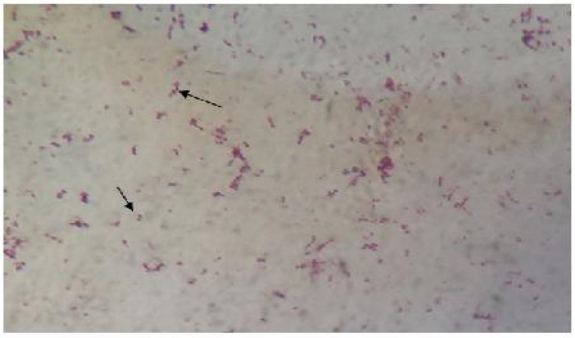

Figure 2 Gram staining showing coco bacilli gram negative pleomorphic rods under 100X (oil impression).

Biochemical test for *Yersinia enterocolitica*

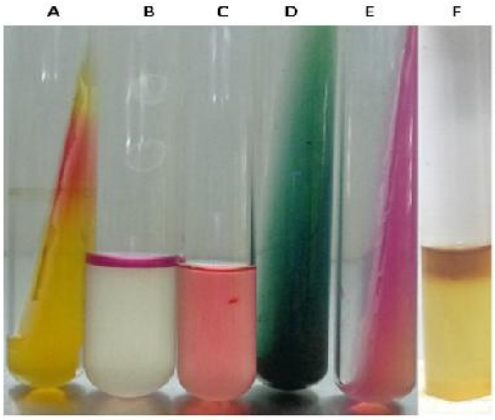

Figure 3 A- Triple sugar Iron agar, B- Indole test, C – Methyl Red test, D – Citrate utilization test, E – Urease test, F - Voges- Proskauer test.

Catalase test for *Yersinia enterocolitica*

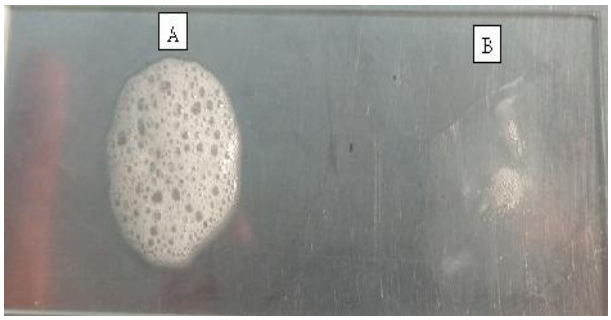

Figure 4 A- Catalase positive reaction liberating bubbles of oxygen. B - Catalase negative reaction.

The results of the present study are summarized in the Table 2. In this present study, an overall prevalence of *Y. enterocolitica* from cow milk and fecal samples was 25 per cent (30/120) which comprising of 10% (5/50) of cow milk and 35.71% (25/ 70) of fecal samples from dairy lactating cows. The higher degree of prevalence of *Y. enterocolitica*in this present study reveals serious issues of milk borne contamination and there is a chance of cross contamination from fecal samples to milk with respect to the public health point of view.

The findings of this study in relation to contamination of bovine milk samples with *Y. enterocolitica* is lower than those observed by other authors, as 29.3% (Subha *et al*, 2009), 36.6% (Rahimi *et al*, 2013), 24.1% (Toora *et al*, 1989). Lower prevalence of *Y. enterocolitica* in milk than the present study was reported by Haifian and Khani (2012) as 7.62%. Some authors failed in detection of *Y. enterocolitica* in milk samples (Ramesh *et al*, 2002 and Zeinhom and Abdel- Latef, 2014). This variation in prevalence may be associated with different factors such as season of the study, geographic location, number of samples and hygienic conditions in the farm and sanitary condition of udder.

The presence of *Y. enterocolitica* in 10% of tested milk samples indicates that the degree of pathogen contamination in bovine milk is higher, it is also reveals consumption of these raw milk and their products has major public health importance because these pathogen capable of grow at refrigerator temperature and some people may consume raw milk because of perceived health benefits.

The main way of spreading sources for *Y. enterocolitica* are human and animal feces and cross contamination with water and some foods. National survey on fecal carriage of *Y. enterocolitica*in pigs, cattle and sheep in Greart Britain during 1999 - 2000 has been conducted by McNally *et al* (2004) and reported 25% of pigs, 10% of sheep and 6% of cattle carried *Y. enterocolitica*. Fukushima *et al* (1983) isolated *Y. enterocolitica* and *Y. pseudotuberculosis* from feces of 618 dairy cows in Japan. In our study 27 (38.37%) lactating dairy cow fecal samples out of 70 were positive for *Y. enterocolitica* by culture and biochemical test. The prevalence of *Y. enterocolitica*in dairy cow fecal samples in this present study was higher than McNally *et al* (2004) and Falcao *et al* 2003, Floccari *et al* (2000). The variation in the prevalence may be due to number of samples, improper hygiene and poor animal management.

In general, pigs are thought to be the major cause of the spreading of pathogenic *Y. enterocolitica*. However, in our study we chosen the milking dairy cows because there are no published reports on prevalence of *Y. enterocolitica* in dairy cows in Tiruapthi region, Andhra Pradesh, South India and also milk will be consumed by all age group people especially children and seniors because it has all nutrients required for body for rapid growth. Hence it is important to screen the dairy animals for presence of emerging zoonotic pathogen like *Y. enterocolitica*. The authors concluded that the isolates from raw milk do not originate in the mammary gland, main source of contamination being feces and/ or contaminated stable (Fukushima *et al*, 1998). However in this study, 10 % of milk samples shown positivity for *Y. enterocolitica*, this may be due to cross contamination from fecal samples of dairy animals or contaminated environment and 38.37% of fecal samples shown positive for *Y. enterocolitica*, hence there is a potential chance for cross contamination of milk samples with these fecal samples.

Table 2 Prevalence of *Yersinia enterocolitica* in bovine raw milk and faecal samples in Tirupathi region, India.

Source	No. of samples examined	Number of samples		Percentage	
		Positive	Negative	% Positivity	% Negativity
Bovine raw milk	50	5	45	10	90
Bovine faecal samples	70	25	45	35.71	64.29
Total	120	30	70	25	75

Antibiogram for Y. enterocolitica

Table 3 shows the antibiogram pattern of the 30 *Y. enterocolitica*, isolated from cow milk and fecal samples, using 14 antibiotics. Twenty eight (93.33%) of the isolates were sensitive to Co-trimaxazole and Amoxycillin, 17 (56.67%) were sensitive to Ampicillin and Tetracycline, 14 (46.67%) were sensitive to Gentamicin, 10 (33.33%) were sensitive to Vancomycin, Chloramphenicol, Ciprofloxacin and Enrofloxacin, 9 (30%) were sensitive for Azithromycin, 2 (6.67%) were sensitive to Oxacillin, 1 (3.33%) were sensitive to Penicillin and none was sensitive to Erythromycin and Streptomycin. Antibiotic susceptibility profile showed that all the isolates were resistant to more than one antibiotic and no isolates were susceptible to all the antibiotics.

However, 29 (96.67%) of the *Y. enterocolitica* isolates were resistant to Streptomycin and Penicllin, 28(93.33%) were resistant to Oxacillin, 13 (43.33%) were resistant to Gentamycin, 8 (26.67%) were resistant to Azithromycin, Erythromycin and Vancomycin, 7 (23.33%) were resistant to Tetracycline, 5 (16.67%) were resistant to Ciprofloxacin and Ampicillin, 4 (13.33%) were resistant to Enrofloxacin, 3 (10%) were resistant to Chloramphenicol, 2 (6.67%) were resistant to Amoxycillin and none was resistant to Co-trimaxazole (Table 3).

In the present study, 93.33% of *Y. enterocolitica* isolates were highly susceptible to Co- trimoxazole which were in accordance with the previous results conducted by Singh and Viridi (2004). Many authors from various regions reported great variation in antibiotic susceptibility pattern of *Y. enterocolitica* isolates may be due to impact of geographical location, difference in the usage of antimicrobials (Fabrega and Vila, 2012). In this study, the isolates were resistant to Co-trimaxazole 0%, Gentamicin 43.33%, Chloramphenicol 10%, Streptomycin 93.33% and Ciprofloxacin 16.67%, whereas in Saleh *et al* (2012) study; it was 37.5%, 68.7%, 62.5%, 87.5% and 43.7%, respectively which is contrast with our study results. However, Subha *et al* (2009) observed that *Y. enterocolitica* isolates were resistant to Tetracycline, Gentamicin, Ampicillin, Erythromycin and sensitive to Co-trimaxazole which is nearly in accordance with the present study. This difference in the results may be due to changes in the antibiotic resistant pattern trends. Studies done in the developing countries showed that *Y. enterocolitica* strains are susceptible to the majority of commonly used antimicrobials (Saleh *et al*, 2012). Our present study results showed that *Y. enterocolitica* isolated from dairy cows were highly resistant to most of antibiotics.

The multiple drug resistance (MDR) patterns are shown in Table 4. Thirty *Y. enterocolitica* isolates elicited 20 different patterns of antibiotic resistance to the agents used in this study (Table 4). The most common multi drug pattern observed in our study was S, P, OX (7/30, 23.33%). Lower number of MDR patterns were reported by Kuan, (2014) as 12 and the highest MDR noted in their study was Nalidixic acid-Clindamycin- Ampicillin- TIC- Tetracycline- Amoxycillin (15/32, 46.9%). Streptomycin,

Table 3 Antibiogram pattern of *Yersinia enterocolitica* from cow milk and fecal samples

Antibiotic used	Concentration(µg)	Antibiogram pattern					
		Susceptible		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Co-Trimoxazole	23.75	28	93.33	2	6.67	0	0
Ciprofloxacin	5	10	33.33	14	43.33	5	16.67
Amoxycillin	10	28	93.33	0	0	2	6.67
Gentamycin	10	14	46.67	3	10	13	43.33
Erythromycin	15	0	0	22	73.33	8	26.67
Vancomycin	30	10	33.33	12	40	8	26.67
Enrofloxacin		10	33.33	16	53.33	4	13.33
Streptomycin	10	0	0	1	3.33	29	96.67
Penicillin	10	1	3.33	0	0	29	96.67
Ampicillin	10	17	56.67	8	26.67	5	16.67
Chloramphenicol	30	10	33.33	17	56.67	3	10
Oxacillin	1	2	6.67	0	0	28	93.33
Azithromycin	15	9	30	13	43.33	8	26.67
Tetracycline	30	17	56.67	6	20	7	23.33

Table 4 Multiple drug resistance pattern of *Yersinia enterocolitica* isolates form dairy cows in Tirupathiresion, Andhra Pradesh, South India

No. of antibiotic resistance	Antibiotic resistance pattern	Source	No. of isolates (%)	Total No. (%) of isolates
Two	S, OX	Fecal	1 (3.33)	2 (6.67)
	S, P	Milk	1(3.33)	
Three	S, P, OX	Fecal, Milk	7 (23.33)	8 (26.67)
	S, OX, TE	Milk	1(3.33)	
Four	S, P, OX, TE	Fecal	1(3.33)	6
	S,P, OX, AZM	Fecal	1(3.33)	
	E, S, P, OX	Fecal	2 (6.67)	
	G, S, P, OX	Fecal	1(3.33)	
	G, VA, S, P	Milk	1(3.33)	
Five	VA, S, P, OX,TE	Fecal	1(3.33)	7 (23.33)
	VA, S, P, OX, AZM	Fecal	1(3.33)	
	E, S, P, OX,AZM	Fecal	1(3.33)	
	G, VA, S, P, OX	Milk	2 (6.67)	
	CIP, G, S, P, OX	Fecal	2 (6.67)	
Six	G, VA, S, P, AMP, OX	Fecal	1(3.33)	1(3.33)
Seven	G, VA, EX, S, P, C, OX	Fecal	1(3.33)	2 (6.67)
	G, E, S, P, OX, AZM, TE	Fecal	1(3.33)	
Nine	CIP, G, S, P, AMP, C, OX, AZM, TE	Fecal	1(3.33)	1(3.33)
Ten	G, E, VA, EX, S, P, AMP, C, OX, AZM	Fecal	1(3.33)	1(3.33)
Eleven	CIP, AM, G, E, EX, S, P, AMP, OX, AZM, TE	Fecal	2 (6.67)	2 (6.67)

Penicillin and Oxacillin antibiotic resistance were most common among the various patterns observed (Table 4). In this study MDR to 3 antibiotics dominated the resistance patterns (8/30, 26.67%) followed by 5 antibiotics (7/30, 23.33%) and 4 antibiotics (6/30, 20%). However in Kuan, (2014) study MDR to 6 antibiotics overtaken the resistance pattern (16/32, 53.33%). The higher rates of multi drug antibiotic resistance in our study indicates alarming situation for designing prevention and control measures. The variation in MDR pattern may be due indiscriminate use of antibiotics may lead to the development of resistance to most currently used antiobiotics and their resistance gene can be transferred to other pathogenic organisms present in gastrointestinal tract (Thong and Modarressi, 2013)

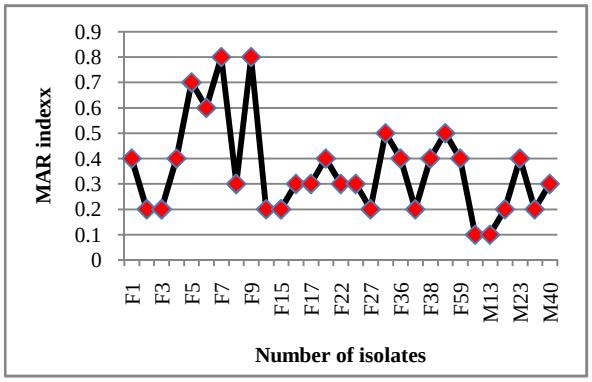

Figure 5 MAR index of *Yersinia enterocolitica* isolated from cows milk and lactating dairy cow fecal samples

Figure 4 showing the pattern of multiple antibiotic resistance (MAR) index for *Yersinia enterocolitica* isolates from cow milk and dairy cow fecal samples.

The MAR index analysis reveals 66.67% (20/30) isolates had a very high MAR index value (>0.2), which implies all these 20 isolates originated from high risk source of contamination. MAR indices less than, or equal to 0.2, identify strains from environment where antibioitcs are seldom or never used (Sahota *et al*., 2014).

In one of the previous study conducted by Subha *et al*., (2009) in milk and pork meat reported that all the isolates (100%) showed a MAR index value of more than 0.2, which is slightly higher than that of our study results.

MAR index of *Yersinia enterocolitica*

CONCLUSION

From the study results that conducted in Tirupathi region, Andhra Pradesh, South India concluded that the presence of *Y. enterocolitica* (10%) in cow milk and lactating dairy cows fecal samples (35.71%) and these positive fecal samples may act as a source of cross contamination to milk. This is clearly indicates that there is a possibility of potential public health threat through consumption of milk and milk products. The level of prevalence can be reduced by adopting hygienic practices in dairy farms. Among the tested antibiotics Co- trimaxazole shown more susceptibility and all the isolates were resistant to more than one drug. The public health significance of this present study is that these resistant strains from milk may find their way into human population through food chain. Hence, there is an urgent need to design the preventive and control measure to prevent the entry of emerging antibiotic resistant *Y. enterocolitica* in food chain by adopting the legislation and enforcement laws.

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