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Determination of prevalence and multidrug resistant *Aeromonas* in raw milk from dairy animals

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Abstract
Background: Food of animal origin diseases are concomitant with high morbidity and mortality and pose a serious threat to public health world over. The increasing occurrence of multiple drug resistant bacterial species is also a matter of concern thereby hindering food safety.
Aim: This study was carried out to determine the prevalence and multidrug resistance phenotypes of *Aeromonas* species in raw milk collected from urban and peri urban areas of Navsari city of South Gujarat.
Methods: A total of 210 samples, contained 70 raw milk each of cow, buffalo, and goat milk were collected using random sampling methods and analyzed as per standard microbiological procedures, Recovered 44 isolates identified and confirmed by targeting *16srRNA* gene as *Aeromonas.* Additionally, isolates were subjected to antibiogram assay using 12 selected antibiotics by agar disc diffusion method.
Result: The out of 210 raw milk samples examined, 44 samples were positive for *Aeromonas* with a prevalence of 21%, and each isolates amplified the *16S rRNA* gene unique to the *Aeromonas* genus. Further 33 (15.7%) isolates amplified the *16Sr RNA* gene that was unique to the *A. hydrophila*, while the remaining 11 (5.23%) amplified isolates were identified as the *A. caviae* species. All the 44 isolates tested for antibiotic sensitivity showed the highest levels of susceptibility to chloramphenicol (95.5%), ciprofloxacin, and nalidixic acid (90% each), next to ofloxacin (86%) and ceftriaxone (81%). Also, isolates were moderately sensitive to co-trimoxazole (75%) and trimethoprim (69%). Isolates exhibited moderate to complete resistance phenotypic pattern to sulfamethizole (30%), gentamicin (52%), tetracycline (40%), ampicillin (52%), and highly resistance to penicillin (100%), each isolate displayed a MAR index between 0.08 and 0.5.
Conclusion: This study advocates sensible and rational use of the antibiotics in the field, so as to safe guard nature and protect human health and the environment in the long run.

Keywords: Milk, *Aeromonas*, *16Sr RNA*, antibiotics, sensitivity, resistant, MAR index

Introduction
Milk is a complete food for all age groups and an essential nutritional demand for healthy diet. But owing to fraudulent practices, water is regularly added as adulterant in raw milk. As a result, pathogenic bacteria are frequently detected in raw milk and its products, which serve as a vehicle for their growth and reproduction. *Escherichia coli*, *Salmonella, Aeromonas*, and *Yersinia* species are some of the most common milk-borne bacterial zoonotic diseases. Direct contact with infected herds and unhygienic conditions on the farm facilitate penetration of *Aeromonas* species into udder tissues, where they multiply and are then released into milk during milking. Post-pasteurization contamination may occur due to the ubiquitous presence of the microorganism. The *Aeromonas* species Gram-negative, facultatively anaerobic, oxidase and catalase positive, non-spore forming, rod-shaped, bacteria capable of converting nitrates to nitrites and ferment glucose (Martinez-Murcia *et al*., 2016) [21]. The genus *Aeromonas* consists of 32 species (Beaz-Hidalgo *et al*., 2015; Marti and Balcazar, 2015 and Martinez-Murcia *et al*., 2016) [9, 20, 21], and 19 species of which are regarded as emerging. They can cause a variety of illnesses in both immunocompetent and immunocompromised patients, such as gastroenteritis, wound infections, bacteremia / septicemia, and respiratory tract infections. As per various studies conducted throughout the world, it has been observed that *Aeromonas* exhibit resistance to commonly used antibiotics as reported by Adams *et al*. (1998) [3] and Rhodes et al. (2000) [24].

Material and Methods
Sample collection
The samples were collected using random sampling method for a period of seven months

between October: 2021 – April: 2022. A total 210 milk samples comprising of 70 each from cattle, buffalo, and goat were collected from urban and peri urban areas of Navsari city of South Gujarat.

Isolation and identification

The modified version of the conventional isolation technique was used and *Aeromonas* species was isolated from milk samples. Each milk sample was thoroughly mixed aseptically, then 1 ml was added to 9 ml of Alkaline Peptone Water (APW, pH 8.6), which were then incubated for 24 hours at 37 °C to enrich the mixture. After that, samples were plated on Ampicillin Dextrin Agar (ADA) that had Ampicillin Dextrin Selective Supplement. The suitable suspected colonies was selected and inoculated on Triple Sugar Iron agar (TSI) and Nutrient Agar plates before moving on to the biochemical analysis. The suspected *Aeromonas* isolates were subjected to standard biochemical characterization tests like Gram staining, catalase test, oxidase test, mannitol motility, indole production, and voges-proskauer test, as well as tests for the production of acids from inositol, maltose, mannose, D-mannitol, sucrose, and cellobiose.

Molecular confirmation

DNA extraction: The isolates confirmed on basis of biochemical tests were cultured in Luria-Bertani broth and incubated at 37 ºC for 24 hours. This was followed by extraction of bacterial DNA, by hot and cold method, which was stored at -20 °C for PCR study.

Polymerase chain reaction (PCR)

The PCR amplification was performed in the total volume of 25-µl reaction mixture consisting of 1 µl of each primer (20 pmol/µl), 12.5 µl of PCR Master Mix, 5.5 µl RNA-free water, and 5 µl DNA Template in a PCR tube. The PCR products were separated on agarose gel (2%) stained with ethidium bromide (5 µg mL^{-1}) applying the run at 90 V voltages for 10 min and 70 V for 1hour. The sizes of DNA fragments were estimated using a DNA ladder (100 bp).

Primers

The PCR amplifications were performed using the following genus specific primers: F (5′-CGA CGA TCC CTA GCT GGT CT-3′) and R (5′-GCC TTC GCC ACC GGT AT-3′) for amplification of the *16Sr RNA* gene with an expected amplicon length of 461 bp. The PCR cycling protocol was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 40 sec, annealing at 63 °C for 50 sec, extension at 72 °C for 40 sec, followed by final extension at 72 °C for 5 min and maintained at 4 °C (Persson *et al*., 2015) [22].

The *A. hydrophila* species-specific primer: F (5'-GGC CTT GCG CGA TTG TAT AT-3') and R (5'-GTG GCG GAT CAT CTT CTC AGA-3') with expected amplicon length of 103 bp. The cycling protocol was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55.5 °C for 30 sec, extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 5 min and maintained at 4 °C (Aboyadak *et al*., 2015) [2].

The *A. caviae* and *A. trota* species-specific primer: F (5'-CTG CTG GCT GTG ACG TTA CTC GCAG-3') and R (5'-TTC GCC ACC GGT ATT CCT CCA GATC -3') with expected amplicon length of 260 bp. The cycling protocol was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 1 min, extension at 72°C for 1 min, followed by a final extension at 72 °C for 5 min and maintained at 4 °C. The fragments of *16Sr RNA* gene region amplified at 260 bp were suspected for *A. caviae* and *A. trota* and were digested with restriction enzyme *AluI* (Thermo scientific FastDigest *AluI*) at 37 °C for 15 min. The reaction was standardized in PCR tubes in 30µl reaction volume with different concentrations of reactants described as follows: PCR product (DNA templet) 10µl, DNase - RNase free water 17µl, Green buffer 2µl and *AluI* enzyme 1µl and The digested products were analyzed by standard submarine gel electrophoresis on a 2% agarose gel at initially at 30V for 10 minutes then 20V for 1 hour and after that 10V for 1 hour. Digested amplicon size yielded 180 and 80bp for confirmation as *A. caviae* species (Khan and Cerniglia 1997) [16].

Determination of antimicrobial resistance

The Kirby-Bauer agar disc diffusion method, as recommended by the Clinical Laboratory Standards Institute (CLSI), was used to determine the antibiogram of the isolates. According to their mode of action and application in clinical therapy, a total of 12 antibiotic discs were selected. penicillin, ampicillin, and ceftriaxone were among the antibiotics that prevent the manufacture of bacterial cell walls. Ofloxacin, ciprofloxacin, and nalidixic acid are among those that prevent the synthesis of nucleic acids. Gentamicin, tetracycline, and chloramphenicol are examples of antibiotics that prevent the production of proteins. sulfamethizole, trimethoprim, and co-trimoxazole are antibiotics that prevent the synthesis of folate. *Staphylococcus aureus* ATCC 25923 was used to evaluate the quality of each antibiotic.

Results

In the current study, a total of 44/210 (21%) *Aeromonas* isolates from raw milk samples were confirmed to be positive. 11/44 samples were classified as an *A. caviae*, while 33/44 samples were positively identified as *A. hydrophila*. (Table 1). To identify the isolates' unique characteristics, each was subjected to a routine biochemical and carbohydrate fermentation test. Isolates that were Voges-Proskauer positive, did not ferment cellobiose, and displayed distinctive fermentation patterns with mannitol were classified as *A. hydrophila*, whereas isolates that were Voges-Proskauer negative but fermented both cellobiose and mannitol were classified as *A. caviae*, as shown in (Table 2). 44 isolates were identified by molecular testing using an *Aeromonas* genus-specific primer (*16Sr RNA*), which amplifies 461 bp, as shown in Figure 1. The 103 bp band was amplified by 33 strains, were found to be *A. hydrophila*, as showed in Figure 2. For confirmation of *A. caviae*, 260bp amplicon was amplified using primers targeting the *16Sr RNA* gene and endonuclease digestion with *AluI* restriction endonuclease enzyme. Isolates were confirmed as *A. caviae* species once they yielded 180bp and 80bp fragments. If there is no fragment after digestion of isolates, they were confirmed as *A. trota*, whereas 11 isolates conformed as *A. caviae*, as depicted in Figure 3 and Figure 4. According to the recent findings, all Aeromonas isolates were (100%) resistant to penicillin, (52%) resistant to ampicillin, and (40%) resistant to tetracycline. The highest level of sensitivity was towards chloramphenicol

(95.5%), ciprofloxacin and nalidixic acid (90% each) followed by ofloxacin (86%) and ceftriaxone (81%) Also, isolates were sensitive to co-trimoxazole (75%) and trimethoprim (69%). A varying degree of intermediate resistance was observed to gentamicin (52%) and sulfamethizole (30%) showed in Table 3. MAR index value of less than or equal to 0.2 considered as the origin of strain from animals in which antibiotics are seldom or never used. Among the strains of *A. hydrophila*, 21.21% were resistant to only one antibiotic, 27.27% were resistant to two medicines, and 39.39% were resistant to three antibiotics. 9.09%, 18.18%, and 54.54% of *A. caviae* strains were resistant to one antibiotic, two antibiotics, and three antibiotics, respectively.

Table 1: *Aeromonas* species prevalence in raw milk samples of dairy animals

Sr. No.	Animals	No. examined	*No. positive Aeromonas* Isolates	Total prevalence of Aeromonas	Spp. of Aeromonas	
					A. hydrophila	*A. caviae*
1.	Cattle	70	16	22.8%	14(20%)	2(2.8%)
2.	Buffalo	70	13	18.5%	7(10%)	6(8.5%)
3.	Goat	70	15	21.4%	12(17.1%)	3(4.3%)
Total		210	44	21%	33(15.7%)	11(5.23%)

Table 2: Conventional biochemical tests of the isolated *Aeromonas* species

Aeromonas spp[a]	Biochemical test										
	Oxidase	Catalase	Motility	Indole	VP	Inositol	Maltose	Mannose	Mannitol	Sucrose	Cellobiose
A.hydrophila (33)	+	+	+	v	+	-	+	+	v	+	-
A. caviae (11)	+	+	v	v	-	-	+	v	+	+	+

Note: a = The total number of isolated colonies was 44 isolates,
+ = 100% positive,
– = 100% negative,
V = Variable

Table 3: Antibiogram of *Aeromonas* isolates

Sr. No	Mechanism of action	Antibiotics	Disc Concent-ration (mcg)	Zone diameter interpretative standard (mm)			N = 44 isolates		
				S	I	R	S (%)	I (%)	R (%)
1	Cell wall synthesis inhibitors	Ampicillin(AMP)	10	29	14-16	28	14.0	34.0	52.0
		Penicillin G (P)	10	29	-	28	00.0	00.0	100
		Ceftriaxone(CTR)	30	23	20-22	19	81.0	18.0	00.0
2	DNA gyrase inhibitors	Ofloxacin (OF)	5	18	15-17	12	86.0	14.0	00.0
		Ciprofloxacin(CIP)	5	21	16-20	15	90.0	9.0	00.0
		Nalidixic acid (NA)	30	19	14-18	13	90.0	4.5	4.5
3	Protein synthesis inhibitors	Gentamycin (GEN)	10	15	13-14	12	21.0	52.0	27.0
		Tetracycline (TE)	30	15	12-14	11	54.0	6.0	40.0
		Chloramphenicol (C)	30	18	13-17	12	95.5	4.5	00.0
4	Folate synthesis inhibitors	Sulfamethizole (SM)	300	17	13-16	12	45.0	30.0	25.0
		Trimethoprim (TR)	5	18	16	11-15	69.0	31.0	00.0
		Co-Trimoxazole (Trimethoprim-sulfamethoxazole) (COT)	25	16	11-15	10	75.0	16.0	9.0
Where: S - Sensitive, I - intermediate, R - Resistance									

Table 4: MAR index value of *Aeromonas* species

Isolates no.	*Aeromonas* species	Resisted antibiotics	MAR index
1	*A. hydrophila*	P	0.08
2	*A. hydrophila*	P	0.08
3	*A. hydrophila*	P, SM	0.1
4	*A. hydrophila*	P, AMP	0.1
5	*A. hydrophila*	P, GEN	0.1
6	*A. hydrophila*	P	0.08
7	*A. hydrophila*	P	0.08
8	*A. hydrophila*	P, AMP, NA, GEN, TE,COT	0.5
9	*A. caviae*	P, AMP, NA, SM	0.3
10	*A. caviae*	P, AMP, SM	0.2
11	*A. caviae*	P, AMP, TE	0.2
12	*A. hydrophila*	P, TE	0.1
13	*A. caviae*	P, GEN, TE	0.2
14	*A. caviae*	P, AMP, SM	0.2

15	*A. hydrophila*	P, AMP, TE	0.2
16	*A. caviae*	P, AMP, GEN	0.2
17	*A. hydrophila*	P, AMP, GEN, TE	0.3
18	*A. hydrophila*	P	0.08
19	*A. hydrophila*	P, TE	0.1
20	*A. hydrophila*	P, GEN, TE	0.2
21	*A. hydrophila*	P, AMP, COT	0.2
22	*A. hydrophila*	P, AMP, SM	0.2
23	*A. hydrophila*	P	0.08
24	*A. hydrophila*	P, AMP, TE	0.2
25	*A. hydrophila*	P, TE, SM	0.2
26	*A. hydrophila*	P, AMP, TE	0.2
27	*A. caviae*	P, AMP, TE	0.2
28	*A. hydrophila*	P, TE	0.1
29	*A. hydrophila*	P, SM	0.1
30	*A. hydrophila*	P, AMP, GEN, COT	0.3
31	*A. hydrophila*	P	0.08
32	*A. hydrophila*	P, AMP, GEN	0.2
33	*A. hydrophila*	P, AMP, SM	0.2
34	*A. hydrophila*	P, TE, SM	0.2
35	*A. hydrophila*	P, AMP, GEN	0.2
36	*A. caviae*	P, AMP, GEN, COT	0.3
37	*A. hydrophila*	P, AMP, GEN, SM	0.3
38	*A. hydrophila*	P, GEN, TE	0.2
39	*A. caviae*	P, AMP	0.1
40	*A. hydrophila*	P, AMP	0.1
41	*A. hydrophila*	P, TE	0.1
42	*A. caviae*	P	0.08
43	*A. caviae*	P, TE	0.1
44	*A. hydrophila*	P, AMP, GEN	0.2

Note: a = antibiotics (AMP- Ampicillin, P- Penicillin, SM- Sulfamethizole, GEN- Gentamycin, NA- Nalidixic acid, TE- Tetracycline, COT- Co-Trimoxazole)

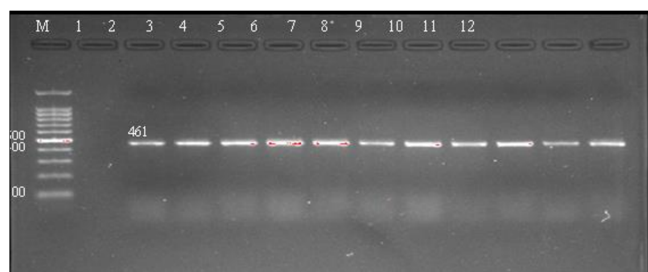

Fig 1: 2% Agarose gel electrophoresis of PCR product showing specific Aeromonas bands at 461 bp. Lane M, 100 bp DNA ladder; Lane 1, negative control (*S. aureus* ATCC 25923); Lane 2-12, pcr product of positive isolates amplified with 461 bp.

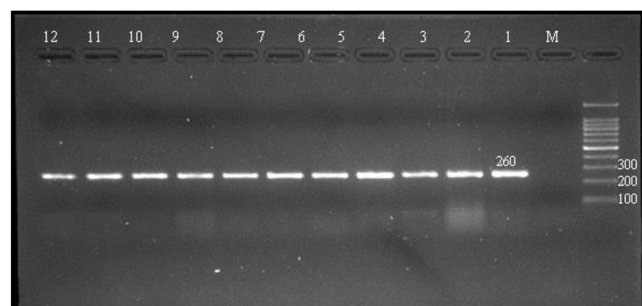

Fig 3: Agarose gel showing PCR amplified product of 260bp for *16Sr RNA* gene of *A. caviae* isolates. Lane M, 100 bp DNA ladder; Lane 1, negative control (*S. aureus* ATCC 25923); Lane 2-12, pcr product of positive samples amplified with 260 bp.

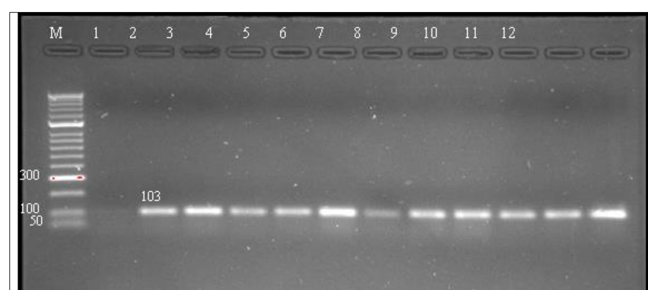

Fig 2: Agarose gel showing PCR amplified product of 103bp for *16Sr RNA* gene of *A. hydrophila* isolates. Lane M, 50 bp DNA ladder; Lane 1, negative control (*S. aureus* ATCC 25923); Lane 2-12, pcr product of positive samples amplified with 103 bp.

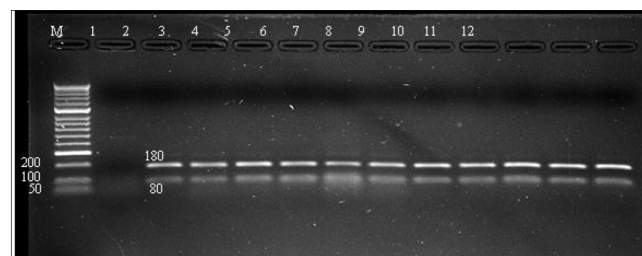

Fig 4: Agarose gel showing digested PCR amplified product of 180 and 80 fragments with *AluI* enzyme for *A. caviae* conformation. Lane M, 50 bp DNA ladder; Lane 1, negative control (*S. aureus* ATCC 25923); Lane 2-12, pcr product of positive samples digested with 180 and 80 bp.

Discussion

Thus in the present study, enrichment in APW followed by selective plating on ADA demonstrated positive growth of *Aeromonas* species, validating the conclusions of Havelaar *et al*. (1987) [13], who replaced starch with dextrin and developed the new medium Ampicillin Dextrin Agar (ADA) as a better selective medium for isolation of *Aeromonas* species. Yucel *et al*. (2005) [30] reported 27% isolates using APW enrichment and ADA from milk and milk products in Ankara. The present study was very close to the observation made by Tahoun *et al*. (2016) [26]. Who reported prevalence as 20%, Enany *et al*. (2013) [12] as 26% and Ibrahim and Mac Rae (1991) [14] as 26.6% positivity rate of *Aeromonas* species from raw milk samples. Contrary to the present study Ahmed *et al*. (2014) [5], Didugu *et al.* (2015) [10] observed higher prevalence of 32% followed by Korashy (2006) [19], Yadav and Kumar (2000) [29], Ahmed *et al*. (2021) [5], Eid *et al*. (2013) [11] and Kirov *et al*. (1993) [17] who reported 37.9%, 45%, 55%, 58% and 60%, respectively. The current research found the predominant species in raw milk as *A. hydrophila* similar to observations reported by Yucel *et al*. (2005) [30], Eid *et al*. (2013) [11], Kirov *et al.* (1993) [17], Yadav and Kumar (2000) [29] and Enany *et al.* (2013) [12]. The PCR assay based on *16Sr RNA* gene can be used for rapid detection of *A. hydrophila* isolates. Trakhna *et al*. (2009) [27] and Aboyadak *et al*. (2015) [2] detected twelve *A. hydrophila* strains using PCR assay resulted the amplification of 103 bp. The findings of the current investigation were total 33 (75%) isolates are confirmed as *A. hydrophila* by using similar primer. For confirmation of *A. caviae*, 260bp amplicon was amplified using primers targeting the *16Sr RNA* gene and endonuclease digestion with *AluI* restriction endonuclease enzyme. Isolates were confirmed as *A. caviae* species once they yielded 180bp and 80bp fragments. If there is no fragment after digestion of isolates they were confirmed as *A. trota* as per reported by Khan and Cerniglia (1997) [16]. In the current study, the target area was amplified successfully using this primer pair with product of 260bp amplicon size and yielded 180bp and 80bp fragments after digestion with *AluI* digestive enzyme wherein 11 *A. caviae* isolates were identified by biochemical testing. Enany *et al*. (2013) [12] reported higher prevalence of *A. hydrophila* (40%) and *A. caviae* (37%) by PCR than present study Alhazmi (2015) [6], Didugu *et al*. (2015) [10] and Ahmed *et al*. (2021) [5] reported lower prevalence of 18.6%, 36% and higher of 55% respectively from raw milk samples. in the research of antibiogram study, all 44 *Aeromonas* isolates were found sensitive to chloramphenicol and ciprofloxacin which is in concurrence with the observations of Koehler and Ashdown (1993) [18], Yucel *et al.* (2003) [30], Awan *et al.* (2009) [7], Eid *et al.* (2013) [11], Alhazmi (2015) [6] and Didugu *et al.* (2016) [10] . 100% susceptibility to ciprofloxacin, chloramphenicol and nalidixic acid was observed by Sadek *et al.* (2017) [25] and Kaskhedikar and Chhabra (2010) and 95% susceptibility towards ceftriaxone was recorded by Koehler and Ashdown (1993) [18] and Eid *et al.* (2013) [11]. The *Aeromonas* isolates were resistant to penicillin and ampicillin in current study with 100% and 52% were similar observation reported by Koehler and Ashdown (1993) [18], Bacchil *et al.* (2002) [8], Yucel et al. (2003) [30], Enany *et al.* (2013) [12] and penicillin exhibit universally resistance to *Aeromonads* reported by Awan *et al.* (2009) [7] and Kaskhedikar and Chhabra (2010) [15]. In the present study, 75% isolates were susceptible co-trimoxazole which is higher than that observed by Kaskhedikar & Chhabra (2010) [15] who reported 62% susceptibility. Awan *et al.* (2009) [7] and Bacchil *et al.* (2002) [28] also reported susceptibility to this drug. Sensitivity towards gentamicin was reported by Koehler and Ashdown (1993) [18] and Kaskhedikar & Chhabra (2010) [15]. Tetracycline showed 54% sensitivity to *Aeromonas*, in contrast to 95% sensitivity reported by Koehler and Ashdown (1993) [18] and Awan *et al.* (2009) [7]. ofloxacin showed sensitivity to *Aeromonas* and similar observations were noted by Awan *et al.* (2009) [7] and Kaskhedikar & Chhabra (2010) [15] . In the current study overall 12.12% strains of *A. hydrophila* were resistant to 3 antibiotics which is contrast to Vivekanandhan *et al*. (2002) [28] who found that only 0.74% strains resistant to 3 antibiotics. Rajab (1999) [23] reported that *A. hydrophila* isolates showed MAR range between 0.08 to 0.6 and *A. caviae* showed range between 0 to 0.5 which justify present research findings that shows 0.08 to 0.5 range to *A. hydrophila* and 0.08 to 0.4 ranges for *A. caviae*.

Conclusion

This study aimed to provide information of the prevalence and antibiotic resistance pattern of *Aeromonas* species in the raw milk samples collected from urban and peri urban areas in Navsari city located in south Gujarat region, out of 210 raw milk samples examined, 44 samples were positive for *Aeromonas* with a prevalence of 21%, Antibiotics are commonly used as growth promoter in animal husbandry which has led to the development of antibiotic resistance amongst a large number of bacterial species. The development of multi drug resistant of *Aeromonas* species pose a serious threat to public health, therefore veterinarians working in the area should focus on judicious use of antibiotics. Bringing awareness among the public about the harmful effect of multi drug resistant micro flora will help to safeguard the human as well as animals from such type of threat. Due to the rising risk of infection from *Aeromonas*, various food reservoirs must be investigated, and it is vital to apply the right preservatives to slow or prevent their growth in consumable foods. Before producing various sorts of milk products, dairy farms must implement appropriate sanitary control procedures in addition to using raw milk of good bacteriological quality. A plan should be kept as part of the HACCP system records.

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Conflict of interest

The authors declare that they have no conflict of interest.

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