

Isolation and molecular characterization of *Listeria* spp. from animals, food and environmental samples

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ABSTRACT: A total of 46 *Listeria* spp. were isolated from 719 samples (milk, bulk tank swabs, cheese, feed, water, faeces and the environment) collected from 415 cattle and 304 sheep over 12 months (from February 2007 to January 2008). These isolates were identified by conventional and PCR techniques as belonging to *L. monocytogenes* (17.4%), to *Listeria innocua* (39.1%), to *Listeria seeligeri* 17.4%), to *Listeria grayi* (15.2%) and to *Listeria welshimeri* (11%). No *Listeria ivanovii* were isolated from any of the samples. *Listeria* spp. were not isolated from cheese and bulk tank swabs. With regard to seasonal variations most *Listeria* spp. were isolated in the spring and winter seasons. The eight *L. monocytogenes* isolates were characterized by PCR-RFLP with *AluI* and *Tsp509I*. RFLP typing of the isolates revealed two different profiles with both restriction enzymes. Four and six different profiles were produced in the examination of *L. monocytogenes* isolates with RAPD analysis with HLWL74 and HLWL85 primers, respectively. This is the first report on the genotyping of *L. monocytogenes* isolates from various sources in Turkey. This study has highlighted the need for improved control and epidemiologic strategies to prevent the transmission of *Listeria* spp. to animals and humans.

Keywords: *Listeria*; seasonal variation; PCR-RFLP; RAPD

List of abbreviations

AFLP = amplified fragment length polymorphism; PCR = polymerase chain reaction; PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism; PFGE = pulsed-field gel electrophoresis; RAPD = random amplified polymorphic DNA

Listeria spp. are widely distributed in the rural environment and may in this manner contaminate milk and production plants (Leite et al., 2006). Moreover, it was reported that cattle farms play a bigger role in the spread of *Listeria* between animals or people rather than small ruminant farms (Pritchard and Donnelly, 1999). Ruminant farm animals play a key role in the persistence of *Listeria* spp. in the rural environment via a continuous faecal-oral cycle (Vazquez-Boland et al., 2001). The risk of listeriosis in ruminants increases with poor quality fermented feeds, for example, when dairy cattle are fed with ensilage foods (Donnelly, 2002). Furthermore, *L. monocytogenes* may also contaminate milk from animals with mastitis.

In addition to the quality of silage, other hygiene parameters, ensured by good herd health management, contribute heavily to the microbiological quality of the milk (Sanaa et al., 1993).

Phenotypic methods such as serotyping and phage typing hold certain drawbacks owing to the existence of non-typable strains, and the low discriminative power of such techniques. Therefore, more discriminatory genotypic methods are needed. To this end, ribotyping (Swaminathan et al., 1996), pulsed-field gel electrophoresis (PFGE) (Kerouanton et al., 1998), amplified fragment length polymorphism (AFLP) (Guerra et al., 2002), and random amplified polymorphic DNA (RAPD) (Vogel et al., 2001)

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have been developed. PFGE and RAPD-PCR are the techniques most often used to type *L. monocytogenes* strains (Cocolin et al., 2005). PFGE is one of the most discriminatory methods, but it is time consuming, the five to seven days are needed before results are available and it requires an expensive apparatus (Franciosa et al., 1998). However, the RAPD technique is appropriate for monitoring strains on a wide scale and for determining whole genome diversity (Wagner et al., 1996). A previous report indicated that the genetic diversity of the *inlA* gene might be useful for discrimination among *L. monocytogenes* isolates from foods, animals and environmental samples by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis (Saito et al., 1998).

The aim of this study was to determine the prevalence of *Listeria* spp. in milk and milk products, animals and environmental samples collected from cattle and sheep over 12 months (February 2007 to January 2008) in eastern Turkey, to explain the seasonal variation of *Listeria* spp., and to investigate the genetic heterogeneity among *L. monocytogenes* isolates by PCR-RFLP and RAPD.

MATERIAL AND METHODS

Collection of samples

Over the course of 12 months, six cattle, five sheep farmhouses and cheese manufacturers were monitored for the presence of *Listeria* spp. in Elazig province, eastern Turkey. All the farmhouses had their own flocks and their own cheese manufacturers. In this region, depending on the season, the animals are fed with pasture, hay and compound feed depending on the farmhouse type. The collection of milk samples is executed either mechanically or manually. There were no cases of listeriosis in the flocks during the surveyed period. All sheep flocks had more than 50 sheep and all cattle flocks had more than five head of cattle.

Between February 2007 and January 2008, milk, cheese, bulk tank, water, feed, faeces and environmental samples were collected from cattle and sheep farmhouses once a month throughout a year. All the milk, water and faecal samples taken from five different animals measured at least 50 g or 50 ml, resulting in a total of 250 g or 250 ml. All the feed, environmental and cheese samples collected from each farmhouse were 250 g or 250 ml in size. Also, swabs were used for bulk tanks. Analysis of

all the samples began in less than 4 h. All samples and bulk tank swabs were immediately submitted to enrichment procedures. All samples taken from farmhouses were pooled together and 25 g or 25 ml samples were used for the first step of enrichment. Swabs were directly used in enrichments procedures in a ratio of 1 g to the 10 ml contained in enrichment broth tubes.

Isolation of *Listeria* spp.

Two different isolation methods were used according to origin of the sample. Milk, cheese samples and bulk tank swabs were analyzed according to the procedures of the Association of Official Agricultural Chemists (AOAC) (Anonymous, 1995) and water, faecal, food and environmental samples were analyzed according to the United States Department of Agriculture (USDA) methods (Anonymous, 2008) for the presence of *Listeria* spp. using a selective enrichment and isolation protocol.

All the isolates were subjected to standard biochemical tests such as Gram staining, catalase test, motility at 25 °C and 37 °C, and acid production from mannitol, rhamnose, and xylose. For further confirmation of *Listeria* isolates, other biochemical reactions, β -haemolytic activity, and Christine-Atkins-Munch-Petersen (CAMP) were measured according to Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986). Bacterial isolates were cultured in TSA + YE at 37 °C for 18 h.

DNA extraction and PCR

Genomic DNA was extracted using the method of Bubert et al. (1992). In order to amplify the entire *iap* gene or portions of it from *Listeria* isolates, PCR was performed with Lis1A and Lis1B primers (Table 1). These primers allowed amplification of a 1454 bp fragment of the *iap* gene of the *Listeria* genus (Bubert et al., 1992). PCR protocols were performed as described by Bubert et al. (1992). PCR conditions were as follows: 30 cycles, each at 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 3 min. PCR products were separated on a 1.2% agarose gel which was stained with ethidium bromide. PCR products were visualized under UV light.

L. monocytogenes specific PCR was performed as described by Border et al. (1990). The primers (Table 1) allowed amplification of a 702 bp frag-

ment of the listeriolysin O sequence of the *Listeria* genus. PCR conditions were performed in one cycle at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min 30 s, and one cycle at 72 °C for 7 min. PCR products were separated on a 1.5% agarose gel which was stained with ethidium bromide. PCR products were visualized under UV light.

Random amplified polymorphic DNA analysis

The technique was performed as described previously by Aguado et al. (2004), with a few minor modifications. Briefly, the reaction mixtures were prepared and amplified by a thermal cycler (Techne TC-512, UK). The amplification reactions were followed by a 45-cycle program: The thermal conditions were the following: a first cycle at 94 °C for 4 min; 39 °C for 45 s; 72 °C for 1 min, followed by 43 cycles at 94 °C for 15 s; 39 °C for 45 s; 72 °C for 1 min, and finally one cycle at 94 °C for 15 s; 39 °C for 45 s and 72 °C for 10 min. The HLWL74 and HLWL85 primers (Table 1) were employed for all *L. monocytogenes* isolates. PCR products were separated on a 1.5% agarose gel which was stained with ethidium bromide and visualized under UV light. Randomly selected isolates were analyzed twice and controls were included in all the reactions to ensure reproducibility.

Polymerase chain reaction-restriction fragment length polymorphism

Restriction analyses were performed as described by Rousseaux et al. (2004). A 733-bp *inlA* fragment was amplified with the primers seq01 and seq02 (Table 1). PCR reactions were performed in 50 µl (total volume) according to Rousseaux et al. (2004).

The PCR conditions were: one cycle of 4 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 52 °C for 1 min and 72 °C for 2 min 30 s, and a final extension step of 72 °C for 7 min. The restriction endonucleases *AluI* and *Tsp509I* were selected on the basis of a partial sequence analysis of *inlA* of *Listeria* isolates. These two restriction endonucleases were used independently. PCR-RFLP fragments were separated by electrophoresis on a 2.5% agarose gel. Gels were stained with ethidium bromide and visualized under UV light.

RESULTS

Isolation and identification results of *Listeria* spp.

A total of 46 *Listeria* spp. were isolated from 719 samples (milk, cheese, bulk tank swabs, water, food, faeces and environment). The prevalence of positive samples was 6.4%. These isolates were identified as: *L. monocytogenes* (eight, e.g. 17.4%), *L. innocua* (18, e.g. 39.1%), *L. welshimeri* (five, e.g. 11%), *L. seeligeri* (eight, e.g. 17.4%) and *L. grayi* (seven, e.g. 15.2%). *L. ivanovii* was not isolated in this study. The majority of *Listeria* spp. (84.8%) were isolated from cattle farms and 13% were found on sheep farms. The prevalence of *Listeria* spp. was found to be 9.4% (39/415) in cattle farms and seven isolates were identified as *L. monocytogenes* (from faeces, feed, water and environment), seven were *L. seeligeri* (from milk, feed, water and environment), 17 were *L. innocua*, (from milk, faeces, feed, water and environment), five were *L. grayi* (from faeces, feed and environment) and three were *L. welshimeri* (from feed and water). The prevalence of *Listeria* spp. was found to be 2.3% (7/304) from feed, faeces and the environment of sheep farms and one isolate was identified as *L. monocytogenes*.

Table 1. Primers used in the PCR reactions

Primer	Genes	Sequence (5'-3')	Product (bp)	References
HLWL74	All DNA	ACGTATCTGC		Yoshida et al., 1999
HLWL85	All DNA	ACAACGTGCTC		
seq01 (F)	<i>inlA</i>	AATCTAGCACCACTGTCTGGG	733	Ueda et al., 2005
seq02 (R)		TGTGACCTTCTTTTACGGGC		
Lis1A	<i>iap</i>	ATGAATATGAAAAAAGCAA	1454	Bubert et al., 1992
Lis1B		TTGGCTTCGGTTCGCGTATAA		
LM1	Listeriolysin	CCTAAGACGCCAATCGAA	702	Border et al., 1990
LM2		AAGCGCTTGCAACTGCTC		

Table 2. Isolation and identification of *Listeria* spp. by sample type

Type of sample	Number of samples	<i>Listeria</i> spp. n (%)	<i>L. monocytogenes</i> n (%)	<i>L. seeligeri</i> n (%)	<i>L. innocua</i> n (%)	<i>L. grayi</i> n (%)	<i>L. welshimeri</i> n (%)
Milk	106	2 (1.19)	–	1 (0.19)	1 (0.19)	–	–
Bulk tank swabs	57	–	–	–	–	–	–
Cheese	28	–	–	–	–	–	–
Feces	132	5 (3.8)	1 (0.8)	–	4 (3)	1 (0.8)	–
Feed	132	20 (15.2)	3 (2.3)	4 (3)	5 (3.8)	4 (3)	4 (3)
Water	132	6 (4.5)	1 (0.8)	1 (0.8)	2 (1.5)	–	1 (0.8)
Environment	132	13 (9.8)	3 (2.3)	2 (1.5)	6 (4.5)	2 (1.5)	–
Total (%)	719	46 (6.4)	8 (17.4)	8 (17.4)	18 (39.1)	7 (15.2)	5 (11)

(from the environment), one was *L. seeligeri* (from feed), one was *L. innocua* (from the environment), two were *L. grayi* (from feed) and two were identified as *L. welshimeri* (from feed). Only one isolate from the environment was isolated from sheep farms. Sources of *Listeria* spp. were as follows: 15.2% from feed, 9.8% from environment, 4.5% from water, 3.8% from faeces and 1.9% from milk samples. More detailed information is provided in Table 2.

Seasonal variations

The prevalence of *Listeria* spp. was very low in summer (2.9%) while this prevalence increased in spring and winter (8.7% and 8.4%, respectively). It is difficult to obtain a reliable estimate on seasonal distribution because of the small sample size in this study. *Listeria* spp. was isolated in all months, except for August. The highest number of *Listeria* spp. were recovered in May (14.1% – nine isolates). More detail regarding seasonal variations is given in Table 3.

Random amplified polymorphic DNA analysis results

All *L. monocytogenes* isolates were analyzed with the primers HLWL74 and HLWL85. The HLWL74 primer yielded four different profiles. Four isolates (1, 3, 5 and 7) were associated with Profile 1. Isolates 2 and 4 with two bands were grouped into Profile 2. Also, isolates 6 and 8 generated one band with HLWL74 and were associated with Profiles 3 and 4, respectively. The HLWL85 primer produced six different profiles. Three isolates (2, 5 and 6) with

three bands were grouped into Profile 1, the other isolates (1, 3, 4, 7 and 8) with 1–3 specific bands were grouped into Profile 2 and 6 (Table 4).

The isolates (1, 3, 5 and 7) grouped into Profile 1 originated from cattle farmhouses and were isolated from the environment, feed (5 and 6) and faecal samples in October, December, April and November. The isolates 3 and 7 were isolated from the same cattle farmhouses, while isolates 1 and 5 were isolated from different cattle farmhouses. Profile 2 contained *L. monocytogenes* isolates (No. 2 and 4) from the environment and feed samples and sheep and cattle farmhouses and were isolated from sheep and cattle farmhouses in July and November. Moreover, two isolates (6 and 8) with one band were grouped into Profiles 3 and 4 (Table 4). These isolates came from the same cattle farmhouses and were isolated from water and environmental samples in April and December.

Isolate No. 2 was grouped into Profile 1 and was recovered from a sheep farmhouse and was isolated from environmental samples in July. The other isolates (5 and 6) grouped into Profile 1 came from cattle farmhouses and were isolated from feed and water samples in April. Although isolates 4 and 8 were grouped into different profiles (Profiles 5 and 4), they originated from the same cattle farmhouse; the profiles of the other isolates shared no common properties.

Results of polymerase chain reaction-restriction fragment length polymorphism analysis

Digestion of the amplified *inlA* fragment with the restriction endonuclease *AluI* generated two different profiles. Four isolates (1, 3, 7 and 8) were as-

Table 3. Seasonal variations in the prevalence of *Listeria* spp. isolates

Seasons	Months	Number of samples	<i>Listeria</i> spp.			
			<i>n</i>	%	total	%
Winter	December	62	6	9.7	16	8.4
	January	65	4	6.2		
	February	64	6	9.4		
Spring	March	65	3	4.6	17	8.7
	April	66	5	9.1		
	May	64	9	14.1		
Summer	June	61	4	6.6	5	2.9
	July	54	1	1.8		
	August	55	–	0		
Autumn	September	56	1	1.8	8	4.9
	October	54	4	7.4		
	November	53	3	5.7		
Total		719	46		46	

sociated with Profile 1. These isolates were isolated from cattle farms. Isolates 3 and 7 originated from the same cattle farm, but were isolated in December and November, respectively. Furthermore, isolates 1 and 7 were grouped into Profile 1 and were isolated from environmental and faecal samples. The other four isolates (2, 4, 5 and 6) were grouped into Profile 2 (Table 4). Isolate number 2 was grouped into Profile 2 and came from environmental sam-

ples extracted from a sheep farm in July. Moreover, 4 and 6 belong to the same cattle farm, but these were isolated in November and April. Isolate number 5 was identified on another cattle farm and was isolated from feed in April.

Digestion of the amplified *inlA* fragment with the restriction endonuclease *Tsp509I* generated two different profiles. Five isolates (1, 3, 6, 7 and 8) generated three bands with Profile 1 and four bands

Table 4. Distribution of *flaA* PCR-RFLP types amongst *L. monocytogenes* isolates

Primers and enzymes	Profiles	Number of isolates	Number of bands	Isolates
RAPD primers	HLWL 74	1	4	1, 3, 5, 7
		2	2	2, 4
		3	1	6
		4	1	8
	HLWL 85	1	3	2, 5, 6
		2	1	1
		3	1	7
		4	1	8
		5	1	4
		6	1	3
Restriction enzymes	<i>AluI</i>	1	3	1, 3, 7, 8
		2	3	2, 4, 5, 6
	<i>Tsp509I</i>	1	3	1, 3, 6, 7, 8
		2	4	2, 4, 5

(2, 4 and 5) with Profile 2. All isolates grouped into Profile 1 came from cattle farms, but 6 and 8 were isolated from water and environmental samples in April and December from the same farmhouse. The other isolates (1, 3 and 7) were grouped into Profile 1 (Table 4).

Isolate 1 originated from different cattle farmhouses and was isolated from environmental, feed, faecal samples in October, December and November. Isolates (2, 4 and 5) grouped into Profile 2. Isolate No. 2 came from a sheep farm and was isolated from environmental samples in July. The other isolates (4 and 5) were taken from different cattle farms and were isolated from feeds in December and November.

DISCUSSION

The true situation of listeriosis in cattle, sheep, environment and cheese in Turkey is not known since there have not been comprehensive studies conducted into *Listeria* and listeriosis in this country. Thus, the fact that listeriosis is an important disease for animal and public health, does not receive due attention. Only the food industry and food laboratories deal closely with listeriosis. The eating habits of Turkish people are different from those in western countries. The majority of people prefer to consume traditionally produced foods. Furthermore, most cattle and sheep farms in Turkey do not have adequate hygiene precautions and animals live in a natural environment together with people. Therefore, we aimed to determine the prevalence of *Listeria* spp. in animals, the environment, and food and to examine the relationship among these parameters in eastern Turkey. The prevalence of *Listeria* spp. was found to be 2.3% in feed, environment, water, faeces and milk samples in sheep farmhouses, 9.4% in feed, environment, water, faeces and milk samples in cattle farmhouses and in total, 6.4%. About half of these isolates (56.5%) were found to be pathogenic in both cattle and sheep and 17.4% of the remaining isolates are pathogens in all animals and humans. A large number of studies have reported on the presence of *Listeria* in various animals and foods. The prevalence of *Listeria* reported in Turkey is similar to the prevalence reported internationally (Cetinkaya et al., 1999; Abay and Aydin, 2005; Aslantas and Yildiz, 2003; Ertas and Seker, 2005; Ozbey et al., 2006). Our results reveal a slightly higher prevalence than

what has previously been determined in the same region and in Turkey. Variations among results can be introduced through the injudicious administration of antibiotics, leading to selection for resistant bacteria and the use of different enrichment methods on animals. These results show the need for improved control and epidemiologic strategies to prevent the transmission of *Listeria* spp. to consumers. Furthermore, our results are consistent with previous studies where it was reported that cattle farmhouses are more significant than sheep farmhouses with respect to listeriosis (Pritchard and Donnelly, 1999; Nicholson et al. 2005).

In a study performed in the Elazig province located in the east of Turkey, four *Listeria* spp. isolates were isolated from sheep milk samples but *L. monocytogenes* was not identified (Ertas, 1999). In another study in the same region, 0.58% of sheep faeces were identified as *L. monocytogenes*-positive (Kalender, 2003). In the present study, samples collected from sheep (faeces, feed and environment) revealed a *Listeria* spp.-positivity of 2.3%, but only one isolate from the environmental samples was identified as *L. monocytogenes*. Silage is not generally used for feeding sheep in the region from where the samples were collected in this study; instead sheep are fed with dry feed and some green grass.

Many researchers have identified raw milk as a source of *L. monocytogenes*, but environmental and faecal contamination during the transportation of milk and its storage have also been reported (Bemrah et al., 1998; Frece et al., 2010). In our study, except for two strains that were isolated from milk, all the other isolates came from faeces, feed, water and environmental samples. In this study, 28 bulk tank swabs were examined, but there was no positive isolation. However, Leite et al. (2006) reported the isolation of *Listeria* spp. from dairy equipment and bulk milk tanks. Although *L. monocytogenes* was not isolated from milk, cheese and bulk tank swabs in this study, *L. innocua* and *L. seeligeri* isolated from milk may reveal possible contamination risks. In the present study, it was observed that the risk of *Listeria* spp. increases during the storage and transportation of milk or because of insufficient standards of hygiene which has been reported in previous studies. Having contaminated milk, *Listeria* infections of animals and people are then transmitted between the environment and food (Nightingale et al., 2004).

Previous studies on *Listeria* spp. prevalence in raw milk and faeces reported some evidence of seasonal variation (Gaya et al., 1996; Ryser, 1999; Abou-

Eleinin et al., 2000; Hutchinson et al., 2005). These seasonal variations were found to be statistically significant in two studies carried out on raw goat milk, in which more samples positive for *Listeria* spp. were found in the autumn (9.33%) than winter (5.14%) and on cattle faeces which was more likely to be positive in March (56.5%) than June (47.1%) (Gaya et al., 1996; Hutchinson et al., 2005). Abay and Aydin (2005) reported isolation from these matrices of, in the autumn (9.3% and 59%, respectively), and in winter (5.1% and 61%, respectively).

We observed that the prevalence of *Listeria* spp. was higher during the spring (8.7%) and winter (8.4%) compared to the autumn (4.9%) and summer (2.9%). Ryser (1999) have reported that seasonal variations in *Listeria* prevalence may be related to silage feeding, with higher prevalences in months when silage is fed to animals. Moreover, in autumn and winter seasons, feeding was carried out predominantly with cattle feed and silage except for sheep fed by breeders.

In this study, a total 46 *Listeria* spp. were isolated using culture and phenotypic methods. All isolates were confirmed by *iap* gene-specific PCR. Although the *iap* gene-specific primers used in this study are very specific, the obtained band profile were not clear. Hence, we hypothesise bacterial genomes may have significantly mutated since the *iap* gene-specific primers were first reported by Bubert et al. (1992).

RAPD-PCR methods were used for the molecular epidemiology of *L. monocytogenes* strains isolated from cheese (Wagner et al., 1996), poultry, and pork plants (Chasseignaux et al., 2001), fish (Ertas and Seker, 2005) and camel sausages (Ozbey et al., 2006). Four and six different profiles were obtained in the RAPD-typing of *L. monocytogenes* isolates with primers HLWL74 and HLWL85, respectively.

Most PCR-RFLP methods have been used to detect polymorphism in the *inlA* region of *L. monocytogenes* isolates from food, animals, plants, the environment and clinical isolates (Schuchat et al., 1993; Wiedmann et al., 1997; Giovannacci et al., 1999). The restriction endonucleases *AluI* and *Tsp509I* provide the best discrimination for RFLP analysis (Rousseaux et al., 2004; Tamburro et al., 2010). In other studies, scientists have reported using the restriction endonuclease *AluI* to study virulence gene polymorphism (Giovannacci et al., 1999; Rousseaux et al., 2004).

In this study, RFLP analysis of the amplified *inlA* fragment of seven *L. monocytogenes* isolates from cattle farms and only one isolate from a sheep farm re-

sulted in two different profiles using the enzymes *AluI* and *Tsp509I*. Three-band profiles of *L. monocytogenes* isolates were observed in six isolates out of eight.

Most studies have reported a much higher genetic heterogeneity of molecular types using both RAPD and PCR-RFLP typing in comparison to what we found in our study (Destro et al., 1996; Rousseaux et al., 2004; Cocolin et al., 2005; Leite et al., 2006; Tamburro et al., 2010). Thus, the use of different primers and more than one random primer or other typing methods may be required for the further analysis of isolates. It was also seen that the number of molecular types returned using RAPD was higher than that of PCR-RFLP. With respect to these findings, the RAPD technique is thought to be a more appropriate method to investigate the genetic relationship between *L. monocytogenes* isolates.

The data reported herein indicate that understanding the effect of seasonal variations can be valuable for the preparation of control programs for interactions among feeds, animals and *Listeria*. More detailed genetic research should be performed to compare *L. monocytogenes* isolates originating from different sources such as animals, feeds and humans and to estimate the listeriosis hazard for animals and humans.

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