The molecular investigation of widespread *Salmonella* serovars, *S. typhimurium* and *S. enteritidis*, involved in salmonellosis of cattle and sheep in farms around Tehran, Iran

Atyabi, N.[1*]; Zahraei Salehi, T.[2]; Ghazisaeedi, F.[1] and Ashrafi, I.[3]

[1]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [2]Department of Pathobiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [3]BSc in Laboratory Science, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[*]**Correspondence:** N. Atyabi, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. E-mail: natyabi@ut.ac.ir

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Summary

Salmonellosis as an important zoonotic disease that causes food born poisoning in human through animal products and is considered as a worldwide public health hazard. Widespread studies have been conducted on different aspects of incidence, treatment and control of salmonelosis all over the world. The aim of this study was to evaluate the occurrence of widespread *Salmonella* serovars, *S. typhimurium* and *S. enteritidis*, isolated from an outbreak of salmonelosis in cattle herds and sheep flocks around Tehran, in summer 2009, using molecular (PCR and multiplex PCR) and conventional (bacterial culture, serology and antibiogram) tests. Tissue and faecal samples were collected from 8 calves, 5 lambs and 2 aborted cattle embryos. All involved cases were animals less than 2-month-old and the presence of *Salmonella* serovars were confirmed in all isolates. The infection of *S. enteritidis* was much more prevalent in comparison to the *S. typhimurium*, which was statistically meaningfull ($P<0.05$). Virulence gene (*spv* gene) of *S. enteritidis* was shown on 250 bp fragments in most of the organ isolates. Specifically the virulence gene was shown in all isolates of aborted fetus tissue cultures, through the molecular survey. In two calves, both *S. typhimurium* and *S. enteritidis* were detected and *S. typhimurium* was isolated from liver in both cases. All isolates were sensitive to streptomycin, lincospectin, enrofloxacine and trimetoprim and were resistant to doxycycline and erythromycin.

Key words: *S. typhimurium*, *S. enteritidis*, Multiplex PCR (mPCR), Antimicrobial sensitivity

Introduction

Salmonella infection is one of the main animal husbandry problems throughout the world. In other words, it is an important zoonotic disease, which can cause food born poisoning in human through animal products, particularly poultry, cattle and pig products (Aragaw *et al*., 2007; Arsenault *et al*., 2007).

Salmonella has more than 2500 different serovars with various host ranges like human and animals including cattle, sheep (Graziani *et al*., 2008), and pig, of which approximately 240 serovars were isolated from the latter (Boyen *el al*., 2008). Some serovars like *S. typhimurium*, *S. enteritidis*, *S. typhi* and *S. paratyphi* cause worldwide public health problems for instance; *S. enteritidis* was recognized as the biggest bacterial food born poisoning pathogen in the USA, especially through the poultry industries (Abasht *et al*., 2008). Both *S. typhimurium* and *S. enteritidis* serovars which have various host range are the main causes of food born infection in Europe and the USA (Graziani *et al*., 2008; Mahé *et al*., 2008). *Salmonella typhi* and *S. paratyphi A*, which are involved in enteric fever are widespread pathogens, especially in Asian countries like China, India, Vietnam, Nepal and Bangeladesh (Wilde, 2007).

Another point to consider is the result of using antibiotics against *Salmonella*

infection in human and animals. Antimicrobial resistance to salmonellosis has occurred in recent years (Aragaw *et al*., 2007), and is a serious problem in the treatment of *salmonella* infection, for instance antimicrobial resistance has caused an increase in *S. typhimurium* infection in Italy (Graziani *et al*., 2008).

The aim of this study was to evaluate the widespread occurrence of the *Salmonella* serovars (*S. typhimurium* and *S. enteritidis*), their pathogenicity and antimicrobial resistance through an outbreak of salmonellosis in cattle and sheep flocks, around Tehran, using bacterial culture, serology, antibiogram tests, PCR and multiplex PCR method as the most efficient way of detection for *Salmonella* serovars.

Materials and Methods

Sample collection

During a salmonellosis outbreak in several farms around Tehran, sampling was performed to identify the molecular characteristics of the causative agent of the outbreak. In this way, whole bodies and tissue samples from 8 calves, 5 lambs and 2 aborted fetuses of cattle, were referred to the Large Animal Hospital of Veterinary School, University of Tehran. The samples were highly suspected to salmonellosis. Because of severe clinical signs, necropsy was performed on all animals except two calves from which just faecal samples were prepared. A total of 44 tissue samples (liver-12, lung-4, bone marrow-9, stomach-2, intestine-7) and 10 faecal samples were collected and immediately sent to the bacteriology laboratory in the Department of Clinical Pathology and Department of Microbiology, College of Veterinary Medicine, University of Tehran.

Bacteriological culture and sero-grouping

Bacteriological culture was performed on 44 samples (tissues from calf and lamb, embryonic tissues and faecal samples) as routine procedures, using Selenite F (SF), blood agar (BA) and MacConkey agar plates as primary culture media. Subcultures from SF medium were applied on *Salmonella-Shigella* agar (SSagar) plates after 18 h at 37°C. Suspected colonies on SSagar were examined biochemically using TSI, urea agar, simmon citrate agar, indole medium, SIM medium, MRVP test and lysine decarboxylation (MERCK Company Supplier, Germany) (Carter *et al*., 2004). In order to indicate genus of *Salmonella* among isolates, serogrouping was performed with the Difco antisera (poly valence, and groups A, B, C, D, Vi…) as directed by their protocol (Carter *et al*., 2004).

DNA isolation, polymerase chain reaction (PCR) and multiplex PCR (mPCR) tests

DNA isolation

All samples were cultured on Luria Bertani (LB) medium overnight at 37°C. DNA extraction was performed by boiling procedure (Rahn *et al*., 1992) using one colony of each culture and boiling for 10 min and then spinning at 6000 × g.

Polymerase chain reaction (PCR)

The PCR was performed to indicate *invA* gene to confirm genus of *Salmonella* by the method of Rahn *et al*. (1992). Briefly, the 25 µl PCR mixture contained: 3 µl of extracted DNA, 10 mM Tris-HCl (pH = 8.3), 100 µM each of dATP, dCTP, dGTP, dTTP (Fermentas, Lithuania), 1.25 mM $MgCl_2$, 50 mM KCI, 0.625 Units of AmpliTaq DNA polymerase (Fermentas) and 0.075 µM of each primer. Both positive and negative control were considered in the PCR set where the negative control contained 3 µl distilled water and *S. typhimurium* (ATCC14028) was used as positive control.

Amplification was done by thermocycler (Model TC-512.; Techne, Cambridge, UK), and PCR program was as follows: the initial denaturation step 1 min at 95°C, followed by 35 cycles containing 30 sec at 95°C, 30 sec at 64°C, 30 sec at 72°C and 7 min at 72°C for the final extension.

The PCR products were electrophoresed in 1.2% agarose (Fermentas) for 1 h at 100 V, and then photographed under UV transilluminator after staining with ethidium bromide (Sinnagen, MR7721C, Litvani).

Multiplex polymerase chain reaction (mPCR)

mPCRs were conducted on *Salmonella* isolates for confirmation of *S. enteritidis* and *S. typhimurium*. DNA isolation was done using the same method as previously described.

mPCR for S. typhimurium

The m-PCR was performed to confirm *S. typhimurium* serovar by Rahn *et al*. (1992) method with ST139-s and ST141-as primers specific for *Salmonella* serovars and the Lim *et al*. (2003) method with Rfbj, Flic and Flijb primers for *S. typhimurium* (Table 1).

Polymerase chain reaction was briefly performed with 10 µl of DNA sample, 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH = 8.5, 1 µM of each primer, 200 µM dNTPs (Fermentas, Lithuania) and 1 U of *Taq* DNA polymerase (Fermentas) in a final volume of 25 µl. Amplifications were performed in a DNA thermocycler. The m-PCR protocol consisted of the following steps: the initial denaturation step of 5 min at 95°C; 30 cycles, consisting of 1 min at 95°C, 1 min at 65°C, 30 s 72°C; and a final extension step of 7 min at 72°C for terminal extension.

The positive control was *Salmonella typhimurium* (ATCC14028). In the negative control, the template DNA was replaced with 10 µl sterile distilled water.

*inv*A primers used for all *salmonella* serovars as for *S. typhimurium* are shown in Table 1.

Amplified products were electrophoresed using the same procedure as described above.

mPCR for S. enteritidis

The mPCR was performed to confirm *S. enteritidis* serovar by Soumet *et al*. (1999) and Pan and Liu (2002) method with ST11- and ST14 specific primers for the genus of *Salmonella* and S1-and S4 primers which is related to virulence and specific for *S. enteritidis* and SEFA4 and SEFA2 primers for specificity within *S. enteritidis* (Pan and Liu, 2002). Polymerase chain reaction was briefly performed by adding 10 µl of extracted DNA of each isolate to 10 mM Tris-HCl (pH = 8.5), 250 µM dNTP (Fermentas, Lithuania), 1.5 mM $MgCl_2$, 50 mM KCl, one U of *Taq* DNA polymerase (Fermentas), and 0.5 µM of each primer in total 25 µl volume. Both positive and negative control were put in PCR set where the negative control contained 10 ml distilled water instead of DNA template and *S. enteritidis* (ATCC13076) was used as positive control. Primers for *S. enteritidis* are shown in Table 1.

Amplification was carried out by the thermo cycler, and PCR program was as

Table 1: Primers used for the detection of *S. typhimurium* and *S. enteritidis* by PCR and mPCR

Target sequence	Primer	Sequence	Amplification product (bp)	Reference
invA	ST139-s ST141-as	5'-GTGAAATTATCGCCACGTTCGGGCAA-3' 5'-TCATCGCACCGTCAAAGGAACC-3'	284	Rahn *et al*. (1992)
Rfbj	rfbj-s rfbj-as	5'-CCAGCACCAGTTCCAACTTGATAC-3' 5'-GGCTTCCGGCTTTATTGGTAAGCA-3'	663	Lim *et al*. (2003)
fliC	flic-s flic-as	5'-ATAGCCATCTTTACCAGTTCCCCC-3' 5'-GCTGCAACTGTTACAGGATATGCC-3'	183	Lim *et al*. (2003)
Flijb	flijb-s flijb-as	5'-ACGAATGGTACGGCTTCTGTAACC-3' 5'-TACCGTCGATAGTAACGACTTCGG-3'	526	Lim *et al*. (2003)
Random[a]	ST11 ST14	5'-GCCAACCATTGCTAAATTGGCGCA-3' 5'-GGTAGAAATTCCCAGCGGGTACTGG-3'	429	Soumet *et al*. (1999)
Spv[b]	S1 S4	5'-GCCGTACACGAGCTTATAGA-3' 5'-ACCTACAGGGGCACAATAAC-3'	250	Soumet *et al*. (1999)
SefA[c]	SEFA2 SEFA4	5'-GCAGCGGTTACTATTGCAGC-3' 5'-TGTGACAGGGACATTTAGCG-3'	310	Woodward and Kirwan (1996)

[a] Randomly cloned sequence specific for the genus of *Salmonella*, [b] *Salmonella* plasmid virulent gene, and [c] *S. enteritidis* fimbrial gene

follows: 35 cycles containing; 30 sec at 94°C, 90 sec at 56°C, 30 sec at 72°C and 10 min at 72°C for final extension.

Amplified products were electrophoresed using the same procedure as described above.

Antimicrobial resistance testing

While mPCR were applied to all isolates, in order to achieve the result, antimicrobial resistance testing was done on proved *salmonella* isolates using the disk diffusion method.

Antibiotics disks (Padtan Teb, Co. Tehran, Iran) containing enrofloxacin (NFX:5 µg), streptomycin (S:10 µg), tetracycline (T:30 µg), gentamicin (GM:10 µg), lincospectin (LP:15/200 µg), doxycycline (D:30 µg), cephalexin (CN:30 µg), ampicillin (AM:10 µg), neomycin (N:30 µg), and cotrimoxazole or (sulfamethoxazole-trimethoprim) (SXT: 400/80 µg) were used to investigate antimicrobial sensitivity.

Resistance evaluation of isolates was tested with the criteria protocol of National Committee for Clinical Laboratory (NCCL) (2003). Standard procedure which is currently called Clinical and Laboratory Standards Institute (CLSI).

Results

According to the bacteriological cultures, biochemical tests, *Salmonella* serogrouping, and PCR tests (Fig. 1) the presence of *Salmonella* serovars was confirmed in all isolates. The outbreak of salmonellosis noticeably happened at the end of the spring and during the summer. All involved cases were animals less than 2-month-old. They showed classical form of salmonellosis, except for two cases where salmonellosis caused abortion in cattle.

The results of serological test and mPCR assay on isolates from tissues and faecal samples, showed that *S. enteritidis* was the only isolate from all samples, except for the liver tissues of two calves with mix infection, which were positive for *S. typhimurium*, and other tissues were positive for *S. enteritidis*. This indicates that *S. enteritidis* (*Salmonella* group D) infection was much more prevalent compared to *S. typhimurium* (*Salmonella* group B) infection (Figs. 2 and 3). In cases with severe infection the disease was generalized and *salmonella* agent was detected from different organs.

In this outbreak, calves involvement was much greater compared to lambs and it was statistically significant ($P<0.05$). Therefore, it seems that the calves are more susceptible to these *Salmonella* serovars than lambs. On the other hand, *S. typhimurium* serovar was only detected from calf samples.

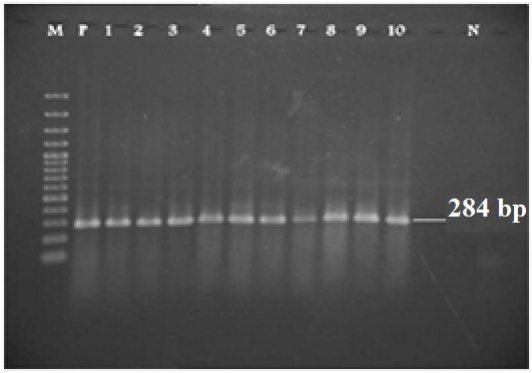

Fig. 1: The presence of *Salmonella* serovars was confirmed in all isolates. Lane M: 100 bp marker, Lane P: Positive control *S. typhimurium* (ATCC14028), Lane 1-10: Isolates of tissues and samples in which the presence of genus of *Salmonella* were shown with primers ST139-ST141 in 284 bp, and Lane N: Negative control

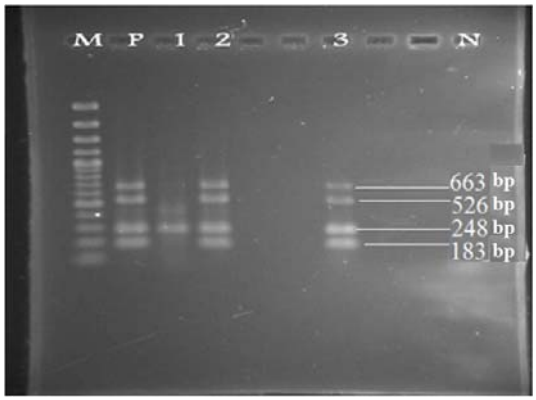

Fig. 2: The *S. typhimurium* infection was confirmed with mPCR assay. Lane M: 100 bp marker, Lane P: Positive control *S. typhimurium* (ATCC14028), Lane 2-3: Isolates which were *S. typhimurium* serovar with Rfbj (663 bp), Flic (183 bp) and Flijb (526 bp) primers for *S. typhimurium*, Lane 1: An isolate which is not *S. typhimurium* serovar, and Lane N: Negative control

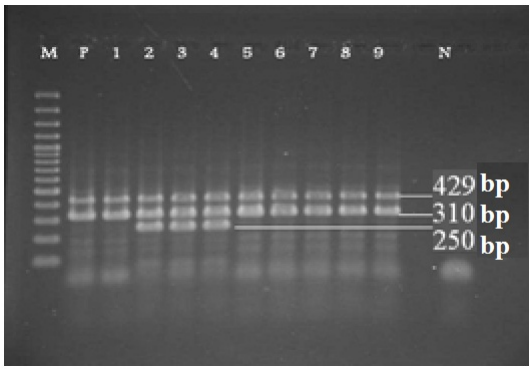

Fig. 3: The *S. enteritidis* infection was confirmed with mPCR assay. Lane M: 100 bp marker, Lane P: Positive control *S. enteritidis* (ATCC13076), Lane 1-9: Isolates of tissues and samples which were *S. enteritidis* serovar with SEFA4, SEFA2 primers for specificity within *S. enteritidis* in 310 bp, Lane 2-4: Virulence gene (*spv* gene) of *S. enteritidis* in 250 bp, and Lane N: Negative control

Virulence gene (*spv* gene) of *S. enteritidis* was shown as a 250 bp fragment in some of the tissue isolates (Fig. 3). Specifically, virulence gene was shown in all isolates of aborted fetus tissue cultures through the molecular survey.

In two calves, both *S. typhimurium* and *S. enteritidis* were detected and *S. typhimurium* was isolated from liver in both cases.

Antimicrobial sensitivity and resistance (Table 2) showed that all isolates were sensitive to streptomycin, lincospectin, enrofloxacine and trimetoprim and resistant to doxycycline and erythromycin. *S. typhimurium* isolates were resistant to ampicillin as opposed to *S. enteritidis* isolates. Only two isolates of calf samples were resistant to neomycin (isolates No. 1 and 3) and one sample was resistant to cephalexin (isolate No. 6). Largely, the antibiotic resistance profile of *S. enteritidis* isolates was almost the same, but in a few cases it was different. Isolates from lamb samples were more sensitive to tetracycline and neomycin than isolates from calves.

Discussion

Recent studies in different countries have been carried out in order to investigate characterization, prevalence, epidemiology, pathogenesis and control of endemic serovars of *salmonella* isolates. For example; Boyen *et al*. (2008), in a recent study in Belgium, performed an experiment on non-typhoidal salmonelosis in pigs. Aragaw *et al*. (2007) showed the presence of an extensive range of *Salmonella* serovars in different organ tissues and faecal samples in slaughtered pigs in Ethiopia. Our experiment was performed to investigate epidemiology, antimicrobial resistance and molecular detection of *S. typhimurium* and *S. enteritidis* in calf and lamb.

Mahé *et al*. (2008) during an experiment in France, estimated the prevalence of salmonelosis by *S. typhimurium* and *S. enteritidis* serovars in laying-hen, indicating the importance of these pathogen serovars in different species of animals all over the world, and in preventing *salmonella* infection in man. Arsenault *et al*. (2007) conducted a research on risk factor and prevalence of salmonelosis in chicken and turkey flocks in Canada.

PCR and mPCR are two efficient methods which are approximately used in all microbial studies for precise identification of pathogens. Zahraei Salehi *et al*. (2007) carried out a study that was intended to detect and identify *S. typhimurium* in bovine faecal samples by molecular assay, which is in agreement with the results of our research. Gillespie *et al*. (2001) performed a PCR-ELISA assay for identification of *Salmonella* serovars.

The present study showed that calves and lambs are more sensitive to salmonelosis than adults. As a matter of fact, calves salmonellosis may be easily transmitted to a flock of sheep, because some of the sheep flocks with *Salmonella* outbreak were in contact with cattle.

The range of influence between cattle herds with positive *S. Dublin* was studied in a recent research in Denmark (Ersbïl and Nielsen, 2008). Atyabi *et al*. (2008) reported chronic extensive peritonitis accompanied by netropenia. Calf faecal sample was serologically positive for *Salmonella* group D.

Jamshidi and Naghdipour (2011) during an investigation using mPCR method, reported that contamination of chiller water to *Salmonella* sp. which was used for chilling process of poultry carcasses, was

Table 2: Antimicrobial resistance profile of isolates was tested with the criteria protocol of National Committee for Clinical Laboratory Standards (2003)

LP	GM	AM	NFX	CN	SXT	N	D	E	S	T	Disk/Sample
I	R	I	S	S	S	R	R	R	S	I	**Calf 1**: Bone marrow
S	R	R	S	I	S	I	R	R	S	R	Liver
I	R	I	S	S	S	R	R	R	S	I	Faecal sample*
I	S	R	R	I	S	S	S	S	S	I	**Calf 2**: Bone marrow
R	S	R	R	I	S	S	S	R	S	I	Liver
I	S	R	R	I	S	R	S	I	S	S	Lung
I	S	R	R	S	S	S	S	S	S	I	**Calf 3**: Bone marrow
R	S	R	R	S	S	S	S	S	S	I	Liver
I	S	R	R	S	S	S	S	S	S	S	Intestine
R	S	R	R	I	S	S	S	S	S	I	**Calf 4**: Bone marrow
R	S	R	R	I	S	S	S	S	S	I	Liver
R	S	R	I	I	S	S	S	S	S	I	Intestine
I	I	R	R	I	S	S	S	S	S	I	**Calf 5**: Faecal sample
I	S	R	R	I	S	S	S	S	S	I	Liver
I	S	R	R	I	S	S	S	S	S	I	Bone marrow
R	S	R	R	S	S	S	S	S	S	S	**Calf 6**: Faecal sample
R	S	R	R	S	S	S	S	S	S	S	Liver
R	S	R	R	S	S	S	S	S	S	S	Intestine
I	S	R	R	S	S	S	S	S	S	I	**Calf 7**: Faecal sample
I	S	R	R	S	S	S	S	S	S	I	**Calf 8**: Faecal sample
I	S	R	R	I	S	S	S	S	S	I	**2 aborted fetuses**: Liver
I	S	R	R	I	S	S	S	I	S	S	Bone marrow
R	S	R	I	S	S	S	S	S	S	S	Lung
R	S	R	R	S	S	I	S	S	S	S	Intestine
R	S	R	I	S	S	S	S	S	S	S	Stomach
I	S	R	R	S	S	S	S	S	S	I	**Lamb 1**: Bonemarrow
I	S	R	R	S	S	S	S	S	S	I	Liver
I	S	R	R	S	S	S	S	S	S	I	Faecal sample
I	S	R	R	S	S	S	S	S	S	S	**Lamb 2**: Faecal sample
I	S	R	R	S	S	S	S	S	S	S	Liver
I	S	R	R	S	S	S	S	S	S	S	Intestine
R	S	R	R	S	S	S	S	S	S	S	**Lamb 3**: Bone marrow
R	S	R	R	S	S	S	S	S	S	S	Liver
R	S	R	R	S	S	S	S	S	S	S	Faecal sample
I	S	R	R	S	S	S	S	S	S	S	**Lamb 4**: Faecal sample
I	S	R	R	S	S	S	S	S	S	S	Liver
I	S	R	R	S	S	S	S	S	S	S	Lung
I	S	R	R	S	S	S	S	S	S	S	**Lamb 5**: Faecal sample
I	S	R	R	S	S	S	S	S	S	S	Intestine

I = intermediate, S = sensitive, and R = resistant

19.2%. From these, 1.9% was due to *S. typhimurium* and 3.8% was due to *S. enteritidis*.

In our experiment *S. enteritidis* serovar was detected in most of the different tissue samples in systemic infection of salmonelosis, but *S. typhimurium* serovar was detected in the liver of two calves. That means the frequency of *S. enteritidis* is much higher than that of *S. typhimurium.*

McCuddin *et al*. (2008) showed that bovine salmonelosis with *S. enteritidis* could involve the neurosystem due to systemic infection.

A study was conducted to determine the prevalence of Salmonellae contamination of chicken carcasses in a slaughterhouse in a city in central Iran. Bacteriological and serological tests showed that the rate of contamination of tissue to *S. typhimuriumis* is 52.2% and *S. enteritidis* is 12.2% (Bonyadian *et al*., 2007).

In a recent study in the United States by Abasht *et al*. (2008), an immunologic

experiment was performed to understand gene expression in spleen and cecum of chicks involved with *S. enteritidis* infection, in order to clarify disease resistance control genetically.

During a research by Graziani *et al*. (2008), in Italy, *S. typhimurium* antimicrobial resistance was studied on isolates from animal and human. The results showed the isolates were resistant to four or more drugs. Their result was in contrast to our results which showed *S. typhimurium* and *S. enteritidis* were sensitive to antibiotics such as streptomycin and ampicillin, however, isolates in our experiment showed resistance to some other kinds of drugs like erythromycin or doxycycline.

Salmonellosis is one of the most important bacterial infections which directly affects public health and animal husbandry, There is clearly a need for further research on this topic.

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