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Research Article

Occurrence of *Campylobacter* spp., *Salmonella* spp. and *Escherichia coli* in Chicken Carcasses

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Abstract The transmission of Foodborne Diseases (FBD) by bacteria constitute a public health problem in the world. This study aimed to identify the presence of *Campylobacter* spp., *Salmonella* spp. and *E. coli* in broiler chickens' carcasses of Botucatu, SP, Brazil, by means of microbiological and molecular methods. Sixty samples of chilled chickens' carcasses of different brands were randomly collected from supermarkets and meat houses located in both peripheral and central area of the city, from January 2015 to January 2016. Later, *Campylobacter, Salmonella* and *E. coli* was isolated from poultry carcasses by conventional microbiological methods, and confirmed by biochemical and PCR tests. The prevalence was 38.3% of *Campylobacter, Salmonella*, 13.3%, and *E. coli*, 60%.
Keywords *Campylobacter spp.; Chicken carcass; Enteropathogens; Escherichia coli; Foodborne diseases; Salmonella spp.*

1. Introduction

Chicken meat production has expanded significantly in recent years, with Brazil being the second largest producer and the largest exporter worldwide. In addition, poultry consumption has increased globally since considered the cheapest animal protein source. In addition, global poultry consumption has increased considerably since considered as the most economical among animal protein sources. Estimations indicate that over 60% of animal protein consumed comes from chicken meat. Currently, Brazil has a per cápita consumption of 46, 2 (MAPA, 2016).

The contamination of food by pathogens, among them bacteria of the Enterobacteriaceae family, as *Salmonella* spp. and *E. coli*, and the Campylobacteriaceae family, such as *Campylobacter* spp., may cause infection or intoxication in humans. These microorganisms commonly dwell in gastrointestinal tract of animals and humans (Fitch et al., 2005; Nzouankeu et al., 2010). The foodborne disease (FBD) is common in most countries. However, its prevalence has increased exponentially by the massive consumption of poultry and eggs (Wilhelm et al., 2011; Williams et al., 2012).

Campylobacter, *Salmonella* and *E. coli* colonize the gastrointestinal tract of a wide variety of wild and domestic animals. Human consumption of mishandling raw meat is the main propagator of these bacteria (Zhao et al., 2001). This bacterial contamination can occur at various stages along the food

chain, including production, processing and distribution. Several epidemiological studies confirm diseases caused by these pathogens, especially in poultry products (Zhao et al., 2001; Rosynek et al., 2005).

Campylobacteriosis is a disease caused by *Campylobacter* spp. Campylobacter species most common are *C. coli, C. lari*, and *C. jejuni*, being the latter responsible for 90-95% of bacterial gastroenteritis. In humans, gastroenteritis is the most common clinical manifestation. The symptoms are similar to those caused by other enteric pathogens. However, the main complication is the low dose to cause infection. Only the intake of 400 to 500 cells can produces the sickness (Butzler, 2004; Rozynek et al., 2005 Gonçalves et al., 2012). This pathology also appears as a significant cause of Guillain-Barré syndrome, a demyelinating inflammatory polyneuropathy resulting in acute neuromuscular paralysis (Mishu and Blaser, 1993; Silva et al., 2014).

Salmonellosis is caused by *Salmonella* spp. affecting animals and humans. From all serotypes described for *Salmonella enterica*, Enteritidis and Typhimurium are the most frequent cases of human salmonellosis in the world (Galanis et al., 2006; Hendriksen et al., 2009). Clinical manifestations range from mild intestinal signs to septicemia. It is found widely in children, elderly and immunocompetents. Although the diarrhea is the main symptom, the intensity varies among patients. Moreover, abdominal discomfort, cramps, fever, nausea, vomiting and headache are also present (Paixão et al., 2016).

The bacterium *Escherichia coli (E. coli)* is the predominant species in the normal enteric flora of most mammals, and generally as a harmless microorganism. However, some strains are quite pathogenic causing urinary tract infections, septicemia, meningitis and gastroenteritis in humans and animals. The high pathogenicity of *E. coli* is due to several virulence factors. *E. coli* causes enteric and extra-enteric events. Based on pathogenicity, it is classified into different groups: enterotoxigenic (ETEC), enteroinvasivo (EIEC), enteropathogenic (EPEC), enterohemorrágico (EHEC), diffuse adherence (DAEC) and enteroaggregative (EAggEC) (Diaz et al., 2016; Ribeiro et al., 2016).

The prevalence of these bacteria has been reported worldwide. However, official statistics on FBD from developing countries like Brazil are scarce. Studies have primarily described the presence of *Campylobacter* spp., *Salmonella* spp. and E. coli in poultry products (Zhao et al., 2001; Cardinale et al., 2003; Modolo et al., 2005; Ha and Pham 2006; Rall et al., 2009; Carvalho et al., 2010; Silva et al., 2014). Based on these grounds, this research aims to identify the presence of *Campylobacter*, *Salmonella* and *E. coli* in broiler carcasses in Botucatu, SP, Brazil, by microbiological and molecular methods.

2. Materials and Methods

Sixty samples of chilled chicken carcasses of different brands were collected from some retail meat stores and supermarkets located at peripheral and central areas of the city of Botucatu, SP, Brazil, in the period from January 2015 to January 2016. The transport of carcasses to the laboratories was carried out at different times to ensure variety of batches. The samples were kept in containers and transported immediately under refrigeration (4-8°C) in cool boxes to the laboratories of Animal Health Planning and Avian Pathology, both from the Faculty of Veterinary Medicine and Animal Science, Campus Botucatu, UNESP. The project was approved by CEUA with protocol number 35/2015.

For isolation and identification of *Campylobacter* spp. in the laboratory, the packaging of each chicken received a cut at the seal region, and placed for two hours on a sterile beaker to obtain carcass drip. Isolation of the agent was performed by using two procedures: filtration and direct seeding. In the filtration process, 10 ml of liquid sample was placed in each test tube, and centrifuged at 2500 rpm for 5 minutes. The supernatant was filtered with the aid of a cellulose acetate membrane of 0.65 µm in diameter (Sartorius Brasil Ltda). Finally, three drops of the filtrate were plated on agar thioglycolate

(Oxoid Brasil Ltda) containing 20% bovine blood, and incubated at 37°C for 72 hours, under microaerobic (Gerador Microaereofilia CO2 GEN-Oxoid Brasil Ltda).

To direct seeding, an aliquot of previously centrifuged and homogenized sample was seeded on the same agar with the addition of the Buzler selective supplement (bacitracin, novobiocin, cycloheximide, colistin and cefazolin.) (Oxoid Brasil Ltda) and incubated for 48 hours at 42°C, in microaerophilic (Modolo et al., 2005). Typical colonies of *Campylobacter* were separated for their identification through biochemical and molecular tests.

For isolation of *Salmonella* spp., 25 ml of carcasses drip were removed, placed in a sterile plastic bag (WHIRL-PAK) containing 225 ml of peptone water, and then incubated at 37°C, for 24 hours. After this, 1 ml and 0.1 ml were transferred to two test tubes containing 10 ml of selective enrichment media, tetrathionate (Muller-Kauffmann tetrathionate/novobiocin-broth MKTTn) and Rappaport (Rappaport-Vassiliadis medium with soya - RVS broth) (Sigma-Aldrich), respectively.

A further incubation was performed for MKTTn at 37 °C for 24 hours, and for RVS at 42°C for 24 hours. Then, by means of a platinum loop, the incubated material was seeded in a selective culture medium Xylose Lysine Desoxycholate (XLD) (Sigma-Aldrich), and in a Brilliant Green Agar (BGA) (Sigma-Aldrich), as described by (ISO 6579-2002).

For isolation of E. coli, 25 ml of carcasses drip were placed in a sterile plastic bag with 225 ml peptone water for subsequent incubation at 37°C for 24 hours. Finally, with the help of platinum loop, the contents were seeded onto MacConkey Agar and Brilliant Green Agar (BGA) (Sigma-Aldrich), and incubated at 37°C for 24 hours. Characterized colonies of *Salmonella* spp. and *Escherichia coli* were separated for identification via biochemical and molecular tests.

Colonies suspected of *Campylobacter* spp. were examined by phase-contrast microscopy (1000X), Carl Zeiss AG, Germany. The diagnosis was made by observing the following morphologies: curved bacillus, and typical movement of spirillum. After the presumptive diagnosis, a suggesting colony were subcultured in a Tarozzi medium, and finally incubated at 37°C for 72 hours, to obtain inoculum with a density adjusted to the scale of the McFarland tube 1 (3x108 CFU/mL). To observe typical features of micro-organism, different biochemical tests were performed such as catalase, growth temperature at 25°C and 43°C, and growth medium at 1% of glycine and 3.5% of NaCl, hydrolysis of hippurate, H2S production with and without cysteine at 0.02%, tolerance to trifeniltetrazóico chloride (TTC), and resistance to nalidixic acid and cephalothin (Veron and Chatelain, 1973; Modolo et al., 2005; ISO 10272-1:2006).

Characterized colonies of *Salmonella* spp. and *E. coli* were placed into agar Triple Sugar Iron (TSI) and agar Lysine Iron Agar (LIA), Sulfide Indole and Motility (SIM), and urea for screening. Colonies compatible with *Salmonella* spp. and *Escherichia coli* were identified by additional biochemical tests (Holt et al., 1994; BAM/FDA, 2014).

Colonies of Campylobacter spp. previously identified by biochemical tests were confirmed by PCR. The isolates were recovered in thioglycolate agar with 20% of blood at 42°C for 48 hours, under microaerophilic. Then, a portion was diluted with 100 µl of ultrapure water, in order to obtain a population of 109 UFC/ml (tube 4 of the McFarland scale). For DNA extraction the kit Gen Elute Bacterial Genomic DNA (Sigma) was used, according to manufacturer instructions.

Typical colonies of *Salmonella* spp. and *Escherichia coli* for each species were inoculated into brain-heart infusion broth (BHI) for 24 hours at 37°C. Subsequently, these colonies were seeded on MacConkey Agar and Brilliant Green Agar (BGA), and incubated at 37°C for 24 hours. The cultures were used for DNA extraction according to the instructions of the kit Gen Elute Bacterial Genomic

DNA (Sigma). The identification of each isolate was confirmed by PCR using primers specific for the genus *Campylobacter, Salmonella* and *Escherichia coli*.

PCR procedures used to identify Campylobacter are previously described by Harmon et al. (1997). The primer pairs used were pg 3: GAACTTGAACCGATTTG and pg 50: ATGGGATTTCGTATTAAC. The reaction had a final volume of 25µL. To complete the reaction volume, 12µL of Go Taq Green Master Mix (Promega), 2µL (20pmol) of each primer, 1µL of DNA (with a concentration of 5nmol/µL), and 8µL water were used. Amplification was performed in a thermocycler by the following program: initial denaturation at 94°C for 4min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1min, and final extension at 72°C for 7min (Silva et al., 2014). The strain of *Campylobacter jejuni* ATCC 33560, courtesy of the Oswaldo Cruz Foundation (FIOCRUZ), served as a positive control. For its recuperation was used the protocol sent by the Microorganism Laboratory Reference. The electrophoresis in agarose gel (1.5%) was useful for the analysis of amplifications.

The identification process of *Salmonella* spp. followed the procedure described by (Rall et al., 2009). The primers used were *invA1*: TCATCGCACCGTCAAAGGAAC and *invA2*: GTGAAATTATCGCCACGTTCGG. PCR reactions had a total volume of 25µL, comprising 12.5µL of Go Taq Green Master Mix (Promega), 1µL (10 pmol) of each primer, 7.5µL of ultrapure and autoclaved water, and 3µL of DNA sample. Amplification was performed in a thermocycler. The cycle parameters were 94°C for 5 min to initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 60°C for 30 secs, amplification at 72°C for 30 secs, ending with a final extension of 72°C for 4 minutes. The positive control was a strain of Salmonella spp. courtesy of Avian Pathology Laboratory of FMVZ-UNESP. The electrophoresis in agarose gel (1.5%) was performed for the analysis of amplifications.

For *Escherichia coli*, reactions were carried out with primers Eco 2083: GCT TTG ACA TGA TGA CAC AG, and Eco 2745: GCA CTT ATC TCT TCC GCA TT. PCR reactions had a total volume of 25µL (5µL (600ng) of extracted DNA), 2.5µL of each primer, 12.5µL of Go Taq Green Master Mix (Promega), and ultrapure water to make up the final volume. Amplification was performed in a thermocycler with an initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 45 secs, annealing of 57°C for 1 min, and amplification at 72°C for 2 min, with a final extension of 72°C for 10 minutes (Rissato et al., 2012).

Statistical analysis was completed using chi-squared test (χ^2 test) and Fisher´s exact test to compare the association between the prevalence of the studied commercial mark of broiler chicken, and the isolation of each bacterium as well as for comparison between the region of collection and isolation. The significance was 5% ($P < 0.05$) (Zar, 1996).

3. Results

Table 1 shows the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli* in isolates of chicken carcasses from stores at peripheral and central locations of Botucatu. Of the 60 samples from analyzed chickens, 23 (38.3%) showed isolation of *Campylobacter*, 8 (13.3%) of *Salmonella* and 36 (60%) of *E. coli.*

Table 1: *Occurrence of Campylobacter spp., Salmonella spp. and E. coli in chilled chickens from the city of Botucatu, SP, Brazil, 2015*

Bacteria	Samples	Prevalence
Campylobacter spp.	23	38.3
Salmonella spp.	8	13.3
E. coli	36	60.0

Total	*67	60

* Simultaneous isolation

Table 2 shows the prevalence of bacterial isolation on carcasses of different poultry brands from the same locations. The statistical test results indicated that there was no significant association (p <0.05) between the commercial marks and the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli*.

Table 2: *Prevalence of Campylobacter spp., Salmonella spp. E. coli in different brands of 181 broiler chickens in Botucatu, SP, Brazil, 2015*

Brand	*Campylobacter* spp.		*Salmonella* spp.		*E. coli*	
	f (nº)	p (%)	f (nº)	p (%)	f (nº)	p (%)
A	4	6.7	1	1.7	3	5.0
B	3	5.0	2	3.3	8	13.3
C	4	6.7	1	1.7	4	6.7
D	5	8.3	0	0.0	8	13.3
E	2	3.3	2	3.3	8	13.3
F	5	8.3	2	3.3	5	8.3
Total	23	38.3	8	13.3	36	60.0
χ^2	8.5145		5.0703		7.9689	
P (Fisher)	0.1519		0.2852		0.1675	

f: frequency; p: prevalence

Table 3: *Proportion of bacterial isolations of Campylobacter spp., Salmonella spp. E. coli in different brands of broiler chickens, Botucatu, SP, Brazil, 2015*

Brand	*Campylobacter* spp.	*Salmonella* spp.	*E. coli*
A	57.1 b	14.3 b	42.9 c
B	23.1 c	15.4 b	61.5 b
C	36.4 c	9.1 c	36.4 c
D	35.7 c	0.0 d	57.1 b
E	22.2 c	22.2 a	88.9 a
F	83.3 a	33.3 a	83.3 a
Total	38.3	13.3	60.0
χ^2	63.966	40.983	36.009
P (Fisher)	<0.001	<0.001	<0.001

Different letters indicate significant differences

The statistical analysis showed a significant difference among the chicken marks referred to the proportions of the three positive bacteria analyzed (Table 3). In this way, brand F showed higher proportion of positivity to *Campylobacter* spp., then brand A. For *Salmonella* spp. and *E. coli*, brands E and F showed higher positivity.

Table 4: *Prevalence of Campylobacter spp., Salmonella spp. E. coli of broiler chickens from different regions of Botucatu, SP, Brazil, 2015*

Region	*Campylobacter* spp.		Salmonella spp.		*E. coli*	
	f (nº)	p (%)	f (nº)	p (%)	f (nº)	p (%)
Central	9	15.0	3	5.0	14	23.3
Peripheral	14	23.3	5	8.3	22	36.7
Total	23	38.3	8	13.3	36	60.0
χ^2	0.5192		0.2098		1.3580	
P (Fisher)	0.1641		0.2713		0.1071	

f: frequency; p: prevalence

Table 4 shows that there was no association between the sample region of origin and the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli*. However, a greater tendency can be noted in the peripheral region when compared to the central region, possibly by inadequate storage conditions, rotation of stocks, and/or hygienic-sanitary control.

Table 5: *Proportion of bacterial isolations of Campylobacter spp., Salmonella spp. E. coli of broiler chickens in different regions of Botucatu, SP, Brazil, 2015*

Region	*Campylobacter* spp.	*Salmonella* spp.	*E. coli*
Central	33.3	11.1	51.9
Peripheral	42.4	15.2	66.7
Total	38.3	13.3	36 (60.0)
χ^2	1.094	0.639	1.847
P (Fisher)	0.3519	0.5455	0.2051

In addition, there was no significant statistical difference ($p < 0.05$) between the samples obtained in the center of the establishments and the periphery in relation to the proportion of positivity of the three bacteria analyzed. However, numerically, the peripheral region was approximately 60% higher in frequency and percentage of positivity than the central region (Table 5).

Table 6: *Prevalence of bacterial isolations of Campylobacter species in broiler chickens Botucatu-SP, Brazil, 2015*

Bacteria	Positive Samples	% Prevalence
Campylobacter jejuni	12	52,17
Campylobacter jejuni\E. coli	9	39,13
Others	2	8,70
Total	23	100

Table 6 shows the results of isolation of *Campylobacter* according to the classification of detected species *C. jejuni* and *C. jejuni/coli* were present in poultry carcasses in 52.17% and 39.13%, respectively.

The positive control of *Campylobacter jejuni* ATCC 33560, through the primer pg3 pg50, which amplifies a conserved region of the flagellin related to both genes *C. jejuni* and *C. coli*, and generates a product of 480 bp. The isolated *Campylobacter* spp. detected from conventional methodology diagnosis, and confirmed by biochemical proofs, resulted also positive for molecular testing (PCR).

The PCR product appears after using the primer pair encoding *inv* A1 and *inv* A2 genes, yielding 284 bp. The presence of *Salmonella* spp. was confirmed by PCR in 7 of samples. However, 8 (13 3%) were positive to the pathogen by traditional methodology. Outcomes related to *Escherichia coli* in chicken carcasses were confirmed by PCR. Primers were used in all reactions for the highly divergent and specific region of the DNA encoding rRNA 16S and 23S of *E. coli*. (Figure not shown). Likewise, based on the antimicrobials tested and on the PCR assays specific, isolates displayed features linked to multidrug resistance and integron class 1 (data not shown).

4. Discussion

The present study demonstrates bacterial prevalence of three major pathogens on chicken carcasses from some supermarkets and retail stores located at peripheral and central areas of the city of Botucatu, SP, Brazil.

Such an occurrence linked to meat consumption varies considerably. For the genus *Campylobacter* spp., it depends on the number of microorganisms originally present in the carcass, hygienic

conditions of slaughterhouses, storage time in shelf and proper conservation (Carvalho and Costa, 1996; Modolo et al., 2005). In addition, some reports indicate the difficulty of isolating *Campylobacter* spp. related to sensitivity to oxygen, desiccation, heat, and pH (Silva et al., 2014). Variations in *Salmonella* spp. depend on the batch origin (primary infection), sanitary conditions of the slaughterhouse, and cross-contamination during the stages of slaughter, transportation and marketing (Olsen et al., 2003; Rall et al., 2009).

These results agree with those obtained in different countries regarding the isolation of the bacteria analyzed. For example, in Senegal, Africa, a research found 56% of prevalence for *Campylobacter* jejuni, and 96% for *Salmonella* spp. (Cardinale et al., 2003). In Washington, USA, authors reported prevalence of *Campylobacter* spp. in 70.7% of broiler chickens, 4.2% for *Salmonella* spp., and 38.7 for E. coli (Zhao et al., 2001). In Vietnam, Asia, 45% of E. coli was identified, followed by 28.3% of *Campylobacter* spp., and 8.3% of *Salmonella* spp. (Ha and Pham, 2006). In Brazil, related studies also revealed such bacteria in chicken carcasses. Detected 47% positive isolates in *Campylobacter* spp., higher values than those indicated in the present work.

The tests performed at 400 chicken carcasses indicated major presence of the bacterium in the carcass sold in the central region than in peripheral region (Modolo et al., 2005). However, Carvalho et al. (2010) found lower percentage of isolation of these bacteria, 14.2%. Silva et al. (2014) determined occurrences in 61% of samples of chicken droppings, 20% in chicken products for consumption, and 3% in human stool, all linked to this microorganism. Carvalho et al. (2010) pointed out the presence of gene complexes of the extensional citoletal toxin (CDT) in 36.4% of the samples. However, Silva et al. (2014) determined 93.5% of such grouped genes for CDT.

In the case of *Salmonella* spp., official organizations such as the European Centre for Disease Prevention and Control (ECDC) and the Center for Disease Control (CDC) have documented a reduced presence of this bacterium when compared with *Campylobacter* spp., mostly due to different regulations implanted worldwide (ANVISA, 2001). However, the prevalence of 13.33% found in this study is still considered high. Moreover, Rall et al. (2009) cited the presence of the pathogen in 8% of the samples in chickens in Botucatu. These values are lower than those expressed in the present research. These authors also indicate that 70% of the samples were out of microbiological parameters.

A similar study on *E. coli* was conducted on chilled chicken carcasses with the brands available in markets and butcher shops of the city of Campo Mourão, Paraná, Brazil. Brand A showed 12.12%, followed by C and D with 6.06% of the positive samples. Brand B showed no positive samples. The results found in poultry carcasses in the city were 24.2%, lower than those reported in this study. (Rissato et al., 2012).

The results presented indicate a public health problem due to the commercial marks used for this research are extensively available for human consumption. Isolates of *Campylobacter* spp. *Salmonella* spp e *E. coli* detected from conventional methodology diagnosis, and confirmed by biochemical proofs, resulted also positive for molecular testing (PCR). *Campylobacter* spp. isolates did not present clear distinction at biochemical tests.

Strains featured both *C. coli*, with a tolerance of 2`3`5 to Triphenyl tetrazolium chloride, and *Campylobacter jejuni*, when hydrolyzed by hippurate. Therefore, they have been named *Campylobacter jejuni/coli*. Similar behavior has been reported by both studies Modolo et al. (1991) in calves and dogs, with and without diarrhea, and Modolo et al. (2005) in broiler carcasses, Botucatu, SP. Véron & Chatelain (1973) indicated problems associated to this bacterium to achieve taxonomic studies on the genus *Campylobacter*. In addition, further investigations highlight difficulties in its classification, probably due to the presence of a common plasmid on such strains (Bradybury, 1983).

Some authors describe unrevealed samples as false negatives, since some medium components of the *Campylobacter* culture like blood or hemoglobin strongly inhibit PCR (Denis et al., 2001). The high protein levels on liver could also explain this interference due to all samples were stored in Tarozzi mediums. Other researchers as Silva et al. (2014) pointed out unsuccessful efforts to obtain *Campylobacter* material for molecular analysis linked to its culturing hardships. Isolates of *Salmonella* spp. and *E. coli* was confirmed through complementary biochemical tests. Unlike *E. coli*, *Salmonella* spp. did not show typical characteristics in all strains.

Thus, molecular diagnosis is important as a viable and reliable alternative to confirm the presence of food bacteria. Speed, specificity and sensitivity are its main advantages (Rall et al., 2009; Rissato et al., 2012; Silva et al., 2014).

Despite previous studies on the assessed bacteria about pathogenic contamination of commercialized broiler chickens, this research demonstrates a significantly remaining prevalence. This must be still considered a very high-risk factor for public health despite Brazilian food health organizations have warned consumers about FBD. Special attention should deserve *Campylobacter* before the absence of specific regulations to monitor and control it. Low infective doses and its relationship with Guillain-Barré syndrome, a disease that leads to muscle paralyses.

In the present study demonstrates the presence of *Campylobacter* spp., *Salmonella* spp., *E. col*i and in different brands of chicken carcasses of meat Botucatu supermarkets and homes, conventional molecular and microbiological methodology. This presents a danger to public health because these bacteria are considered by international organizations as the main causes of foodborne diseases.

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