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PREVALENCE AND ANTIBIOGRAM PROFILE OF *LISTERIA MONOCYTOGENES* ASSOCIATED WITH CATTLE FARMS IN LAFIA, NASARAWA STATE

Bashir Eya Sani, Femi Gbadeyan, Kabiru Shuaibu, Naja'atu Shehu Hadi and Fatima Al-Mustapha Yusuf*

Address (es):
Department of Microbiology, Faculty of Science, Federal University of Lafia, PMB 146, Lafia, Nasarawa State

*Corresponding author: bashmodulus@gmail.com

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ABSTRACT

Listeriosis, mostly caused by ubiquitous *Listeria* spp., notably *L. monocytogenes* is a foodborne disease of great public health concern with high mortality and severe symptoms. However, it is greatly underreported and diagnosed. Using standard microbiological procedures (EN ISO 11290-12017) and Kirby-Bauer antimicrobial disk diffusion method, the prevalence and antibiogram profile of listerial contaminants from four cattle farms were assessed. A total of 160 samples, 40 samples per farm comprising ten samples each of cattle dung, feed, milk and water were collected. Of the total studied samples, 20.63% (33/160) were positive for listerial contaminants. Farm A (6.88%; 11/160) and cattle feed samples (33%; 13/40) accounted for the highest prevalence among farm and sample type, respectively. Seven different species of *Listeria* were isolated with *L. monocytogenes* being the most prevalent (42.42%; 14/33) and *L. welshimeri* and *L. grayi* the least prevalent (6.06%; 2/33). 28.57% (4/14) of *L. monocytogenes* isolates harbored the *hlyA* virulence gene. A high antimicrobial-resistant rate and MAR index range of 0.07-0.73 to tested antibiotics, including to last-resort drugs, was observed in this study. *L. monocytogenes* isolates were 100% susceptible to Clarithromycin, with high resistance observed to nalidixic acid (100%), streptomycin (64.29%) and ofloxacin (42.86%). This study findings revealed a significant level of listerial contamination in cattle farm samples. The presence of *Listeria* spp., especially antimicrobial-resistant *L. monocytogenes* is a cause for alarm. Appropriate regulatory and monitoring authorities are encouraged to sensitize cattle-breeders and consumers appropriately and step-up checks in the sales of cattle products in Lafia.

Keywords: Listeriosis, *L. monocytogenes*, prevalence, antimicrobial resistance, food safety, listeriolysin, *hlyA* gene

INTRODUCTION

One of the major complex issues that is affecting the society; both government, academia, industry and the general public is food safety. Foodborne diseases caused by microbial pathogens have constituted a global health problem (**Al-Ashmawy *et al*., 2014**). Food contamination is a major problem associated with retailed food and snacks in our society today. There has been observed increased in the rate of people falling sick from consuming contaminated foods due to safety slips, this is despite the increased attention and resources that have been pumped to improve consumer and public health (**Amadi *et al*., 2014**).

Amongst the most prevalent foodborne diseases, is listeriosis which refers to infections caused by *L. monocytogenes* and other species of the genus *Listeria*. Listeriosis has been reported (**Mead *et al*., 1999**) as having the highest hospitalization rate of 91% amongst foodborne pathogens with 400 - 500 deaths said to occur each year and an estimated 2500 persons becoming severely ill as a result of this disease in the United States of America (**Borucki *et al*., 2005**). Listeriosis has also been reported to have a fatality (mortality) rate ranging from 20-30% globally (**Sergelidis & Abrahim, 2009**).

Listeric infections caused by members of the genus *Listeria* occur worldwide, in man and in various animals**(Hood, 1993; Low & Donachie, 1997**). Seven *Listeria* species have been identified, namely; *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. murrayi, L. grayi* and *L. welshimeri* **(Gebretsadik *et al*., 2011**). *L. monocytogenes* and *L. ivanovii* are pathogenic (**Liu *et al*., 2006**), but *L. monocytogenes* is the principal pathogen in humans and animals. *Listeria ivanovii* is a pathogen of animals but is occasionally implicated in human disease. The other *Listeria* species are generally considered non-virulent **(Bille & Doyle, 1991; Low & Donachie, 1997**). However, sporadic human infections due to *L. seeligeri* and *L. innocua* have also been reported (**Perrin *et al*., 2003**). The clinical syndromes of the disease caused by pathogenic species of *Listeria* in humans include invasive listeriosis, non-invasive gastrointestinal disease, and local skin and eye symptoms **(Maijala *et al*., 2001**).

Invasive listeriosis is characterized by severe symptoms including encephalitis, meningoencephalitis, abortion, sepsis with about 30% mortality; while non-invasive listeriosis manifests with several mild symptoms such as abdominal pain and vomiting, diarrhea, nausea, fever and muscle pain (**Lundén *et al*., 2004**). Listeriosis leads to significant loss in production of livestock – especially veterinary animals (such as cattle, sheep, and goats) (**Low & Donachie, 1997**) – through morbidity and high mortality. It also represent an environmental-human infection link as it is of paramount importance to food safety and public health (**Lawan *et al*., 2013**).

L. monocytogenes are Gram-positive, facultative anaerobic intracellular pathogens. They have been reported to adapt a vast range of dynamic conditions, surviving and reproducing in extreme conditions such as low or high pH, temperature and water activity **(Mead *et al*., 1999**). Previous outbreaks of listeriosis have been associated with ready-to-eat food products. It has also been reported that *L. monocytogenes* can survive for months and be shed in the faeces of healthy cows as well as through milk of cattle suffering from mastitis caused by *Listeria* spp. (**Mead *et al*., 1999; Ryser *et al*., 1997**). Contamination of milk can also occur through the use of contaminated equipment or contact with dung **(Arimi *et al*., 1997**).

With its associated high mortality rate and severity of symptoms associated with listeriosis, and increased emergence and concern of multidrug-resistant *L. monocytogenes*, listeriosis is considered a severe health problem. However, the prevalence and antibiogram profile of *L. monocytogenes* are under-documented in Lafia. This work aimed to determine the occurrence and antibiogram profile of *Listeria* spp. in Lafia cattle farms.

MATERIAL AND METHODS

Sample collection

A total of 160 samples, forty (40) each of cow dung, cow feed, water and fresh raw milk were collected from different cattle farms in Lafia, Nasarawa State between May to September 2020. Water and cow feed samples were collected from their respective trough. Fresh and stalled cow dungs were collected randomly from the floor and mixed while fresh raw milk samples were collected directly from the cows' teat. All samples were aseptically collected (avoiding cross contamination) and transported immediately, in less than 30 minutes to the lab in ice packs for microbiological analysis.

Isolation and identification of Listeria isolates

The EN ISO 11290-12017 method (ISO, 2017; Public Health England, 2019) for inoculation and isolation of *Listeria* contaminants from samples was adopted with modifications. Aseptically, 25 grams (for solid samples: feed and dung) or 25 milliliters (for liquid samples: water and milk) of each sample was measured and into 225mL of *Listeria* Enrichment Broth (LEB) comprising *Listeria* enrichment selective supplement (Oxoid, UK). The sample solutions were homogenized with a blender (for solid samples) or vortex (liquid samples) at room temperature for 120 seconds and incubated for 24h at 37°C. After incubation, *Listeria* species were isolated by seeding 1mL of the overnight LEB culture solution unto *Listeria* Selective Agar (LSA) by pour plate method and incubated at 37°C for 48h. Plates were observed for black-green color colonies with black halo and sunken center characteristic of *Listeria* spp.

Identification of *Listeria* spp. was carried out using standard methods (**Chuku *et al.*, 2019**). Gram stain, CAMP (Christie, Atkins and Munch-Peterson), motility, hemolysin production, urease, sugar (rhamnose, xylose, mannitol and mannose) fermentation, catalase and indole tests were the confirmatory tests carried out to identify the presumptive listerial isolates.

Antibiogram profiling and MAR index

The antibiogram profile of the *Listeria* spp. isolated was ascertained using the Kirby-Bauer disk diffusion method on Mueller Hinton Agar (**Clinical and Laboratory Standards Institute (CLSI), 2020**). The isolates were tested against the following antibiotics: chloramphenicol (30µg), ofloxacin (5µg), Tetracycline (30µg), ciprofloxacin (5µg), ampicillin (10µg), nalidixic acid (30µg), fusidic acid (10µg), gentamicin (10µg), sparfloxacin (5µg), erythromycin (15µg), rifampicin (5µg), Clarithromycin (15µg), co-trimoxazole (25µg), Augmentin (30µg), and streptomycin (10µg)

Five discrete colonies of *Listeria* spp. isolates were cultured overnight for 18h in 5mL Luria-Bertani (LB) broth at 37°C, and the turbidity standardized to 0.5 McFarland standard (OD_{625mm}=0.08-0.13; approximately 10^8cfu/mL). Using sterile swab cotton sticks, aliquots of the culture were spread onto freshly prepared and dried Mueller Hinton Agar plates to form a lawn and allowed to stand for 5 minutes. At most four (4) antibiotic discs were placed on each plate's surface and incubated at 37°C for 24 hours. Thereafter, the diameter of clear zone of inhibition around each antibiotic disc was measured in millimeters (mm) using a meter ruler and data retrieved was interpreted according to the CLSI guidelines using *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATC25923 breakpoints (**Clinical and Laboratory Standards Institute (CLSI), 2020**). Multiple antibiotic resistance (MAR) index was determined as the ratio between the number of antibiotics an isolate is resistant to (a) and the cumulative antibiotic it was exposed too (b); mathematically expressed as:

$$MAR\ index = \frac{a}{b}$$

Detection and prevalence of L. monocytogenes harbouring hlyA genes

L. monocytogenes harbouring *hlyA* genes were detected using multiplex PCR as previously described (**Usman *et al.*, 2016**). The primers (LM1 and LM2) were used to detect the *hlyA* genes where: LM1 5′–CCT AAG ACG CCA ATC GAA-3′ and LM2 – 5′-AAG CGC TTG CAA CTG CTC-3′. *L. monocytogenes* ATCC19155 were used as reference strain. A programmed thermocycler was used to carryout PCR amplifications of 25µL reaction mixture with the following conditions: initial denaturation at 95°C for 2 minutes, 35 cycles each of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds), extension (72°C for 1 minute), final extension at 72°C for 5 minutes and storage at 4°C till product was removed for further analysis. The reaction mixture consisted of 5µL of PCR buffer (5x), 2µL each of DNA template and dNTPs, 13µL of distilled water, 0.5µL each of $MgCl_2$, Taq DNA polymerase and 20pM primers (LM1 and LM1).

After PCR amplification, gel electrophoresis of the PCR product was carried out on 1% agarose gel by mixing the PCR product (5µL) with 6x loading dye and electrophoresed at 120v for 20 minutes and visualised under a UV transilluminator.

RESULTS AND DISCUSSION

Prevalence of Listeria spp. isolates

Table 1 shows the number of positive samples of *Listeria* species isolated from the 160 samples. A total of 20.63% (33/160) of the studied samples were positive for *Listeria* spp. Farm A had the highest contamination rate of 6.88% (11/160), while Farm D had the lowest rate of 3.75% (6/160). Cattle dung samples had the highest prevalence of *Listeria* spp (11/40; 28%) while fresh raw milk samples had a prevalence rate of 3% (1/40).

Table 1 Prevalence of *Listeria* spp. contaminants on the different sample types from the studied cattle farms

Cattle sample	Farms				Cumulative Frequency (%) n=40
	A (%)	B (%)	C (%)	D (%)	
Dung (%) n=10	4 (40)	3 (30)	2 (20)	2 (20)	**11 (28)**
Feed (%) n=10	4 (40)	4 (40)	2 (20)	3 (30)	**13 (33)**
Drinking Water (%) n=10	2 (20)	2 (20)	3 (30)	1 (10)	**8 (20)**
Fresh Raw Milk (%) n=10	1 (10)	0 (0)	0 (0)	0 (0)	**1 (3)**
Total (%) N=160	**11 (6.88)**	**9 (5.63)**	**7 (4.38)**	**6 (3.75)**	**33 (20.63)**

Table 2 shows the occurrence and distribution of *Listeria* species isolated from the different cattle farm samples. Of the 33 *Listeria* spp. isolate, *L. monocytogenes* had the highest prevalence rate of 42.42% (14/33), followed by *L. ivanovii* with a prevalence rate of 15.15% (5/33) and *L. welshimeri* and *L. grayi* jointly having the lowest rate of prevalence at 6.06% (2/33). At least one *Listeria* sp. is absent from each sample type. *L. seeligeri* was the only positive *Listeria* isolated from fresh raw milk.

Table 2 Occurrence and distribution of *Listeria* isolates from the different sample types

Sample Type	*Listeria* species (%)						
	L. mon	***L. mur***	***L. iva***	***L. see***	***L. inn***	***L. wel***	***L. gra***
Dung (n=11)	6 (54.55)	0 (0)	3 (27.27)	1 (9.09)	0 (0)	1 (9.09)	0 (0)
Feed (n=13)	6 (46.15)	1 (7.69)	2 (15.38)	0 (0)	2 (15.38)	1 (7.69)	1 (7.69)
Drinking water (n=8)	2 (25.00)	2 (25.00)	0 (0)	1 (12.50)	2 (25.00)	0 (0)	1 (12.50)
Fresh raw milk (n=1)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Total (N=33)	**14 (42.42)[a]**	**3 (9.09)**	**5 (15.15)**	**3 (9.09)**	**4 (12.12)**	**2 (6.06)**	**2 (6.06)**

Legend: *L. mon: L. monocytogenes*; *L. mur: L. murrayi; L. iva: L. ivanovii; L. see: L. seeligeri; L. inn: L. innocua; L. wel: L. welshimeri; L. gra: L. grayi;*
[a]: significantly different at $\rho<0.05$

Antibiogram profile and MAR index of L. monocytogenes isolates

The antibiogram profile – susceptibility, intermediate and resistance – of *L. monocytogenes* isolated from the studied cattle farm samples is as shown in Table 3. A 100% susceptibility and resistance were observed in clarithromycin and nalidixic acid respectively. Isolates were 92.86% susceptible to fusidic acid and ampicillin, 85.71% to rifampicin and 78.57% to Tetracycline. A 64.29%, 42.86% and 35.71% resistance were also observed in streptomycin, ofloxacin and co-trimoxazole respectively.

Table 3 Antibiogram profile of *L. monocytogenes* isolated from the studied cattle farms (N=14).

ANTIBIOTICS	Susceptible (%)	Intermediate (%)	Resistant (%)
CN	9 (64.29)	2 (14.28)	3 (21.42)
ST	3 (21.42)	2 (14.28)	9 (64.29)
TE	11 (78.57)	2 (14.28)	1 (7.14)
SP	8 (57.14)	3 (21.42)	3 (21.42)
RD	12 (85.71)	1 (7.14)	1 (7.14)
PN	13 (92.86)	0 (0)	1 (7.14)
CLR	14 (100)	0 (0)	0 (0)
OFX	6 (42.86)	2 (14.28)	6 (42.86)
SXT	6 (42.86)	3 (21.42)	5 (35.71)
AU	9 (64.29)	2 (14.28)	3 (21.42)
CPX	10 (71.43)	3 (21.42)	1 (7.14)
E	8 (57.14)	4 (28.57)	2 (14.28)
FC	13 (92.86)	1 (7.14)	0 (0)
NA	0 (0)	0 (0)	14 (100)
CH	7 (50.00)	5 (35.71)	2 (14.28)

Legend: CN: gentamycin; ST: streptomycin; TE: Tetracycline; SP: sparfloxacin; RD: rifampicin; PN: ampicillin; CLR: Clarithromycin; OFX: ofloxacin; SXT: co-trimoxazole; AU: Augmentin; CPX: ciprofloxacin; E: erythromycin; FC: fusidic acid; NA: nalidixic acid; CH: chloramphenicol.

The Multiple Antibiotic Resistance (MAR) index of the tested *L. monocytogenes* isolates ranged from 0.07 to 0.73 as shown in Figure 1. DU4 had the highest MAR value of 0.73 (11/15), followed by DR13 with a MAR index of 0.60 (9/15), and DU3 and DU5 both having a MAR index of 0.53 (8/15). DU1, which was resistant to only one (1) antibiotic had the lowest MAR index of 0.07 (1/15) and followed closely by FE12 and DR14 which both have MAR index of 0.13 (2/15). The isolate from milk sample was susceptible to all tested antibiotics.

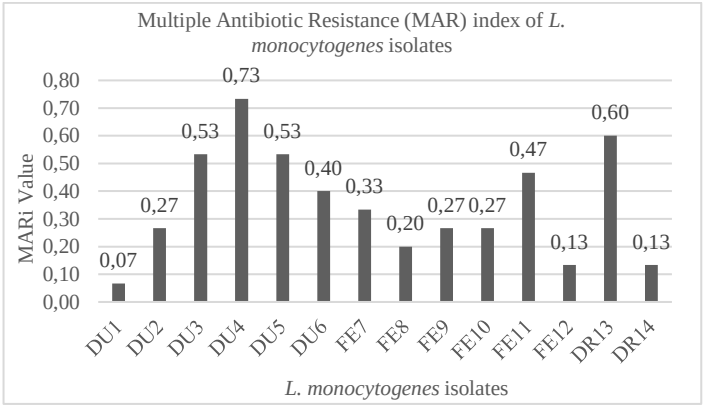

Figure 1 Multiple Antibiotic Resistance index (MAR) of *L. monocytogenes* isolates. DU1-6: Dung samples; FE7-12: Feed samples; DR13-14: Drinking water samples

Prevalence of hlyA harbouring L. monocytogenes

Figure 2 shows the PCR results of *L. monocytogenes* isolates harbouring listeriolysin O (*hlyA*) A gene. Of the 14 identified *L. monocytogenes* isolates, only 4/14 (28.57%) carry the *hlyA* gene.

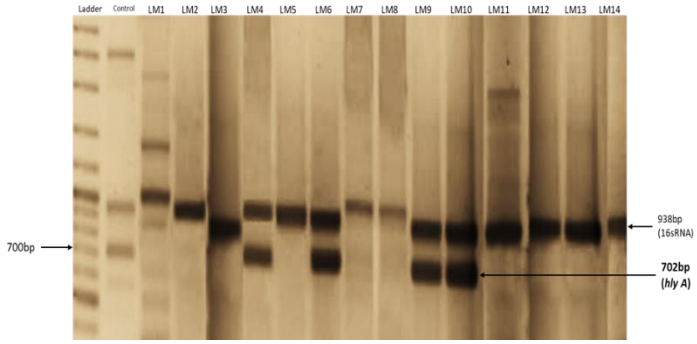

Figure 2 Agarose gel electrophoresis for detection of listeriolysin O gene (*hlyA*: 702bp). Lanes: Ladder: 1000bp; Control: *L. monocytogenes* ATCC19155; LM1-14: *L. monocytogenes* isolates from cattle farms in Lafia

DISCUSSION

Listeric infection – despite its severity, easy of transmission and high rate of mortality – have gained minimal attention from health and other regulatory authorities in Nasarawa State and Nigeria. There is scarce information and no documented report on the epidemiology of *Listeria* and listeriosis in Lafia, especially in vehicles of transmission such as cow meat and milk which are in high demand in the studied location. This study is the first report that assessed the prevalence, antibiogram and distribution of *hlyA* resistant genes of *Listeria* spp. in Lafia cattle farms. The results of this study showed a 20.63% (33/160) prevalence rate of *Listeria* spp. from cattle farm samples in Lafia, Nasarawa. The prevalence rate observed in this study was higher than the 6.4% (**Atil *et al*., 2011**) and 13.2% (**Shourav *et al*., 2020**) prevalence rate earlier reported but lower than the 91.8% prevalence rate in chicken flocks and meat in Oyo State (**Ishola *et al*., 2016**).

Among the seven *Listeria* species isolated, *L. monocytogenes* was the most observed while *L. welshimeri* and *L. grayi* were the least observed. This conforms with the earlier report (**Chuku *et al*., 2019**) on *Listeria* prevalence on fresh beef and chevon but contradicts reports of (**Yakubu *et al*., 2012**) and (**Atil *et al*., 2011**). Cattle feed samples had the highest listerial contaminant rate of 33% (13/40), followed by dung samples with 28% (11/40) prevalence rate with milk samples only having a 3% (1/40) listerial contaminant rate. Amongst the farms studied, Farm D had the lowest contamination rate of 3.75% (6/160) with Farm A having the highest prevalence rate of 6.88% (11/160). Poor facility maintenance and drainage system, illiteracy and unawareness by the cattle-breeders, use of contaminated water, improper storage facilities, drug misuse and abuse and other unhygienic practices observed during sampling could be attributed to the observed result. *Listeria* species' presence portends human and public health danger and concern as they can be transmitted to humans and animals that use or consume cattle products.

Listeriolysin O (*hlyA*) is associated and enhances *L. monocytogenes*' intestinal barrier and cell evasion capability, motility, intracellular parasitism and cell-to-cell spread (**Liu *et al*., 2007**). This study showed that not all (4/14; 28.57%) *L. monocytogenes* isolates were harboring the *hylA* gene. This is in agreement with (**Usman *et al*., 2016**) who had a similar observation of 25% (9/36), but disagrees with findings of (**Jallewar *et al*., 2007**) where all isolates harbored *hylA*. The absence of this virulent gene in some of the isolates could be attributed to spontaneous mutation, or simply because they are environmental isolates**(Témoin *et al*., 2008**).

The study also finds that *L. monocytogenes* exhibited varying degree of sensitivity to the antibiotics tested with a MAR index range of 0.07 to 0.73; indicating a high drug resistance. *L. monocytogenes* isolates were highly susceptible to Clarithromycin (100%; 14/14), and ampicillin and fusidic acid (92.86%; 13/14). The varied susceptibility and multiple antibiotic resistance observed is similar to other reports(**Ndahi *et al*., 2014**). These results are similar to those obtained by **Caplan *et al*. (2014)**, showing that almost all the studied strains were susceptible to a wide range of antibiotics but were completely resistant to nalidixic acid. This study findings also showed that the isolates were totally (100%; 14/14) resistant to nalidixic acid which is in concordance with (**Chuku *et al*., 2019**) but discords with the report of (**Shourav *et al*., 2020**) where about 42% *L. monocytogenes* isolates were susceptible to nalidixic acid. However, this study's findings validate the use of nalidixic acid as supplements in *Listeria* enrichment and selective media. The susceptibility and resistance to gentamicin, ampicillin, erythromycin, chloramphenicol and co-trimoxazole observed in this study is similar to that reported by (**Hansen *et al*., 2005**). The misuse of antibiotics by cattle breeders and

veterinary quacks (**Yakubu *et al.*, 2012**) could be attributed to the observed antimicrobial resistance. The horizontal transfer of antimicrobial-resistant genes and plasmids within and between *Listeria* species and other closely related bacteria (such as *Staphylococcus, Enterococcus* and *Streptococcus*) in the environment could also be responsible for the antimicrobial resistance **(Safdar & Armstrong, 2003**) observed in this study.

CONCLUSION

This study has shown that there is presence of *Listeria* in cattle farm samples in Lafia. Prevalence of *Listeria monocytogenes*, especially strains harboring *hlyA* gene which enhances pathogenicity and virulence, is worrisome considering the high morbidity, mortality, and other peculiar characteristics of listeriosis, especially to the YOPI (young, old, pregnant and immune-compromised) fraction of the population who are the dominant populace in Lafia. Lack of social and health care facilities also endangers the populace as an outbreak of listeriosis will be difficult to manage.

The sensitivity profile is alarming, and as such, caution still needs to be administered in diagnosing and administering drugs to patients with listeriosis as more and more resistant strains of *L. monocytogenes* have now been observed, both from human, clinical, food and other related samples. Cases of abortion, meningitis and septicemia need to be appropriately investigated and treated in neonates, immune-compromised, pregnant and the elderly.

Unhygienic practices amongst the collection site have been identified as the major source of contamination. Use of contaminated water, washing without disinfectant, lack of awareness and improper storage facilities are the major causes of the high contaminant rates observed. Inadequate facilities and equipment maintenance and dirty muddy environment also mediate the observed contamination rate. There is a need for appropriate inspection, sensitization, orientation and regulation by appropriate authorities to check the quality of cattle and their products to reduce the prevalence of listerial contaminants, a possible outbreak of listeriosis and safeguard public health.

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