Original Research Article

Prevalence of multidrug ~~resistance~~ resistant gram-negative bacteria in tissues of diseased chicken in Chitwan District, Nepal.

Abstract

For increasing productivity in poultry, antibiotics are overused. This increased use in antibiotics has raise the prevalence of Multidrug resistant~~ce~~ (MDR) bacteria ~~(MDR)~~ in poultry. Treatment of chicken infected with MDR bacteria ~~are~~ is difficult to ~~treat~~ acieve, thereby increasing treatment cost and productivity cost. MDR bacteria of poultry can also infect humans if they are not handled properly. Thus, the purpose of this study was to find bacteria responsible for infecting chicken and prevalence of MDR bacteria in diseased chicken. Out of total 516 diseased chicken, 212 (41.09%) chicken were infected by bacteria. The prevalence of *E. coli* (63.2%) was high in diseased chicken followed by *Salmonella* spp. (12.26%), *Pseudomonas* spp. (5.2%) and ~~,~~ *Pasteurella* spp. (4.7%). Out of total number of isolates, the prevalence of MDR was 42.5 %. This study also showed that *Pasteurella* spp. isolates had high MDR with prevalence of 50%. It is thus concluded that there was a~~s~~ high prevalence of MDR bacteria among diseased chicken in Chitwan district.

Introduction

Antibiotic resistance is a result of antibiotic use (1). The greater the volume of antibiotics used, the greater will be the chances of arising antibiotic resistance population of bacteria (1). There is growing scientific evidence that the use of antibiotics in chicken feeds leads to the development of resistant pathogenic bacteria that can reach humans through the food chain (2). Recent reports have shown that different types of food and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and food-producing animals (3). Multidrug resistant~~tee~~ (MDR) bacteria ~~(MDR) of bacteria~~ is defined as a bacteria that is resistant~~tee~~ to different classes of antibiotics (three or more than three classes of antibiotics) which are structurally different and have different molecular targets (4). The spread of MDR bacteria outside the hospital environment has posed a serious problem over the last few years, and now poultry with rather extensive use of antibiotics has become a possible source for multi-resistant bacteria (5). Consequently, one possible transmission route for MDR bacteria from animal to a human being is food, especially meat and meat products. Poultry has been recognized as an important source of human infections (5).

Bacterial microorganisms of importance to public health, such as coliforms, especially *Salmonella* and *Escherichia coli* (*E. coli)*, have been found as part of the normal flora in several domestic animals, including chickens (6). Fowl cholera, caused by *Pasteurella multocida*, remains a major problem of poultry worldwide (7). *Pseudomonas aeuroginosa* causes high mortality in newly hatched chickens and ~~was the~~ death of an embryo at a later stage (8). A wide variety of disease conditions are associated with pathogenic organisms ~~that may be~~involving bacterial, viral, parasitic, fungal, mycoplasma and other non-infectious diseases that have always been a threat to the growing poultry industry (9).

In a developing country like Nepal, routine microbiological tests for the detection of the microorganism and its antibiotic susceptibility ~~test~~ are not performed. Due to the prescription of antibiotics by veterinarians without the antibiotic susceptibility test, there is an increase in the resistance of bacteria towards the antibiotic. Thus, the main objective of our study ~~is~~ was to identify the pathogenic bacteria according to breed, determine antibiotic resistance and m~~M~~ultidrug resistance (MDR) pattern of identified bacteria from infected chicken samples.

2. Material and methods:

2.1 Study design:

Cross-sectional study design was used in this study. All the diseased chicken which ~~was~~ were presented in National Avian Disease and Investigation Laboratory (NADIL) from December 2017 to May 2018 were enrolled in the study. Study samples were diseased and dead chicken brought for disease diagnosis. Breeds of chicken enrolled in the study ~~which are~~were layers, broiler, broiler parents and backyard chicken. ~~were enrolled in the study.~~ A total of 516 samples of chicken breeds were included in this study.

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2.2. Sample collection:

Tissues (Liver, lungs, t~~T~~rachea, and h~~H~~eart) were collected based on clinical findings and pathognomonic lesions observed during detailed postmortem examination of poultry at

postmortem section of NADIL according to the chicken breed. Bacterial contaminations were observed according to chicken breed to find out which chicken breed is highly susceptible to gram negative bacteria (*E. coli*, *Salmonella*, *Pasteurella* and *Pseudomonas*). In most frequent form, gram negative bacteria such as *E.coli*, *Salmonella*, *Pasteurella* and *Pseudomonas* are observed in upper respiratory tract, lungs, liver and heart (10, 11) Samples were collected into sterile pPetri dishes in postmortem section and immediately transported in to the microbiology laboratory.

2.3. Isolation and identification of Gram-negative bacteria:

The samples was taken from the diseased chicken and brought in to Avian Laboratory for examination. It wasSamples were washed with 70% alcohol to deplete aerosol contamination. Some portion of the sample was flamed with a red-hot blade. Then swab was taken from the sample and enriched in peptone water and incubated at 37°C°C for 24 hours. The sample was inoculated in nutrient agar and MacConkey agar plate using a standard inoculating loop. The plate was incubated at 37 °C°C for 24 hours. After overnight incubation, the colony was characterized.

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2.4. Microscopic observation:

Microscopic examination was observed by Gram staining method. The organisms revealinged pink-colored colonies with the rod-shaped appearance and arranged in single or in pairs were suspected as *E. coli* (12). If growth was observed in nutrient agar but not in MacConkey agar, then the isolates from nutrient agar wereas again sub cultured on blood agar to confirm the purity of the culture. Pure colonies from blood agar were suspected as *Pasteurella* (13).

Cultural methods for the detection of *salmonella* spp. involved a non-selective pre-enrichment, followed by selective enrichment and plating onto selective and differential agars. After pre-enrichment, 1 ml of enriched cultures of sample types were transferred to 9 ml of selenite f. broth and incubated at 37°c for 18 hrs. A loopful of culture from selenite f. broth was streaked into plates of XLD and were incubated at 37°c for 18 hours (14). The grown colonies on the nutrient agar and Muller- Hinton agar characterized by producing diffusible pigments and sweet grape odor (bluish-green or yellowish-green) were selected for further tests for *P. aeruginosa* (15).

2.5. Biochemical test:

A further biochemical test was performed for the identification of these bacteria. Bacteria were identified by performing standard biochemical tests (SIM test, MRVP test, urease test, citrate test) (16).

2.6. Antibiotic susceptibility test of isolated bacteria:

Clinical and Laboratory Standards Institute (CLSI) recommended Modified Kirby-bauer disk diffusion method was used for antibiotic susceptibility test (17). Place aAgar plates placed right side up in an incubator were heated to 37°c for 10 to 20 minutes with the covers adjusted so that the plates are were slightly opened. Inoculate Aall agar plates were inoculated with their respective test organisms as follow; dip a sterile cotton swab into a well-mixed saline test culture and removes excess inoculated by processing the saturated swab against the inner wall of the culture tube. Allow all culture plates to dry for about 5 minutes. Gently press each disc down with the wooden end of a cotton swab or sterile, forceps to ensure that the discs adhere to the surface of the agar. Finally incubate all plate cultures in an inverted position for 24 hours at

37°C (18). After overnight incubation, the plates were examined for confluent growth. The diameter of the zone of inhibition was measured and interpreted by referring to the zone of diameter. Ciprofloxacin, Gentamicin, Amoxicillin, Amikacin, Cotrimoxazole, Doxycycline and Levofloxacin ~~are~~ were the antibiotics used as they are the antibiotics of choice for treatment of bacteria- infected diseases. ~~For this~~In the present study, antibiotic discs ~~we~~ used were ~~Antibiotic discs~~ purchased from Himedia, India.

Ethical approval and consent to participate: Ethical approval was obtained from Research Ethics Committee of Balkumari College, Tribhuvan University, Nepal. The study protocol was verified by Research Committee of Microbiology Department. No human sample was involved in this study and the animal samples were processed according to the animal research ethical guidelines. Informed written consent was obtained from all poultry farm owners included in the study.

Results

Out of ~~a~~ total ~~of~~ 516 samples, 212 (41.09%) were found to be positive and the rest of them did~~oes~~ not show any growth ~~in~~ on culture media ~~{~~(Fig 1)~~}~~. Furthermore, ~~we~~ the samples were separated ~~our samples~~ according to their breed ~~in~~ as shown in Fig 2. ~~figure 2.~~ Out of 300 ~~layers~~ samples from layers, 114 (38%) samples showed growth whereas 30 (54.54%) broilers out of 55 samples showed growth ~~in~~ on media. In addition, the samples include 51 broiler parents who had~~ave~~ 23 (45%) positive growths and 110 backyards who had~~ve~~ 45 (40.9%) samples that showed growth. Here, Backyards include local chicken, Giriraj, Lohmann, Hyline-brown, etc.

The results have shown that bacterial growth was found to be higher in broiler chicken followed by broiler parents, backyard, and layers, respectively.

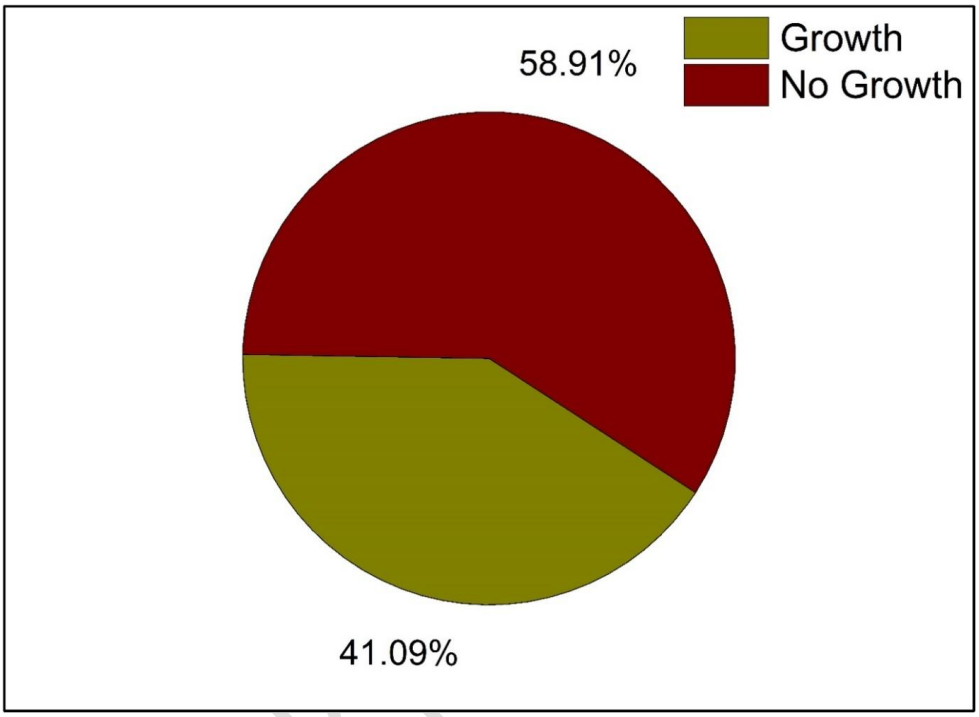

Figure 1: Prevalence of pathogenic bacteria in the total sample

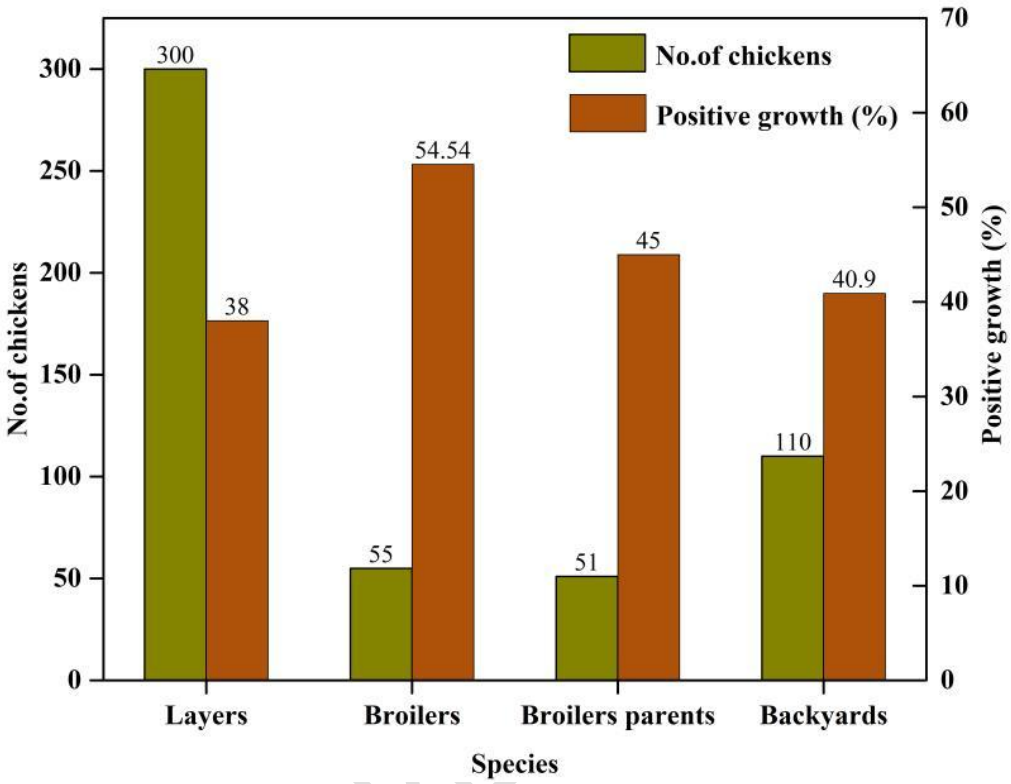

Figure: 2 Prevalence of chicken disease according to breed

The growth of *E. coli* was higher among pathogenic bacteria in all breeds. Out of 212 growth samples, 134 (63.2%) samples had growth of *E. coli*, 10 (4.7%) *Pasteurella* spp., 11 (5.2%) *Pseudomonas* spp., and 26 (12.26%) *Salmonella* spp. The results also pointeds out the growth of other bacteria such as *Staphylococcus* spp., *Kklebsiella* spp., *Campylobacter* spp., *Serratia* spp., etc. which ~~are discarded from our research~~were not included in the present study ~~[~~(Table 1)~~]~~. ~~We~~ ~~f~~Further, the samples were separated ~~the samples~~ according to the division of pathogenic bacteria ~~according to the~~as per breed.~~,~~ *E.coli* were~~are~~ more susceptible to broilers, *Pasteurella* and *Pseudomonas* were~~are~~ susceptible to b~~B~~roiler parents and *Salmonella* were~~are~~ susceptible to b~~B~~ackyards ~~[~~(Table 2)~~]~~.

Table 1: Prevalence of ~~P~~pathogenic bacteria in poultry diseases

Isolated bacteria	**No. of isolated bacteria (%)**
Escherichia coli	134 (63.2)
Salmonella spp.	26 (12.26)
Pseudomonas spp.	11 (5.2)
Pasteurella spp.	10 (4.7)
Others	31 (14.6)
Total Number of isolated bacteria	**212**

Table 2: Prevalence of p~~P~~athogenic bacteria according to breed

Bacteria / Breed	*E. coli (%)*	*Pasteurella* spp. (%)	*Pseudomonas* spp. (%)	*Salmonella* spp. (%).	Others *(%)*
Layers	74 (64.91)	5 (4.38)	6 (5.26)	15 (13.17)	14(12.28)
Back yard	23 (51.11)	1 (2.22)	1 (2.22)	8 (17.77)	12(26.67)
Broiler	21 (70)	0 (00)	2 (6.67)	2 (6.67)	5(16.67)
Broiler parent	16 (69.57)	4 (17.39)	2 (8.7)	1 (4.53)	(00)

We performed an antibiotic susceptibility test of that Pathogenic bacteria using seven common antibiotics (Gentamicin, Cotrimoxazole, Levofloxacin, Amoxicillin, Amikacin, Doxycycline, and Ciprofloxacin). Out of seven antibiotics used in this study, Gentamicin was found to be the most effective against *E. coli* whereas Levofloxacin was found to be least effective. Most of the isolates of *Pasteurella* were susceptible to the Cotrimoxazole whereas resistant to Levofloxacin and Amoxicillin. *Pseudomonas* spp. were sensitive to Gentamicin whereas resistant to Levofloxacin and Amikacin was more effective against *Salmonella* but resistant to ciprofloxacin [Table 3]. Out of total isolates, multidrug resistant of *Pasteurella* were found to be higher (50%) followed by *E. coli (*48.5%), *Pseudomonas (*18.2%) and *Salmonella (*13.9%) [Table 4].

Table 3: Antibiotic susceptibility tests of pathogenic bacteria

Antibiotics used	Isolated Bacteria Zone of inhibition (mm)

	E. coli			*Pasteurella* sp.			*Pseudomonas* sp.			*Salmonella* sp.		
	S %	I%	R%	S%	I%	R%	S%	I%	R%	S%	I%	R%
Doxycycline	22.3	24.6	53.1	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**
Ciprofloxacin	27.9	20.5	51.6	14.3	00	85.7	18.2	27.3	54.5	38.7	13	48.3
Gentamicin	73.3	00	26.3	**NT**	**NT**	**NT**	91	00	9	73.5	00	26.4
Amikacin	71	12.2	16.8	**NT**	**NT**	**NT**	77.8	11.1	11.1	79.8	00	20.7
Levofloxacin	9	11	80	00	00	100	27.3	00	72.7	26.7	40	33.3
Amoxicillin	**NT**	**NT**	**NT**	00	00	100	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**
Cotrimoxazole	**NT**	**NT**	**NT**	28.57	00	71.5	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**

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Table 4: Frequency of multidrug resista~~nn~~t (MDR) e ~~-~~bacteria ~~(MDR)~~

S. N	Bacteria	No. of MDR Bacteria (%)	No. of Non-MDR Bacteria (%)	Total No. isolated bacteria
1	*E. coli*	65 (48.5)	69 (51.5)	134
2	*Salmonella* spp.	5 (13.9)	21(86.1)	26
3	*Pseudomonas* spp.	2 (18.2)	9 (81.8)	11

4	*Pasteurella* spp.	5 (50)	5 (50)	10
Total		77 (42.5)	104 (57.45)	181

Discussion

In this study, *E. coli* isolated from tissues (Liver, tTrachea, and hHeart) of chicken ~~was~~ were 63.2%. The high prevalence ~~is~~ was because *E. coli* have rapid multiplication rate and are predominantly found in excreta of humans and animals (14). Some strains of *E. coli* are acid tolerant which makes it more adaptive to extreme condition (14). *E. coli* can also form biofilm to protect itself from antibiotics, chemical disinfectants, desiccation, predators and ultraviolet radiation (14). The biofilm also provides nutrition to *E. coli* making *E. coli* predominant in environment (14). ~~A~~ In the previous study, ~~done in Pakistan found~~ the prevalence of *E. coli* was reported as ~~of~~ 35.31% (15). Our study shows high prevalence of *E. coli* infection in chicken in Chitwan district. The favorable temperature for *E. coli* is greater than 30° C and Chitwan belongs to subtropical region with temperature range of 7° C to 42.5° C (14, 16). ~~A similar study done in 2016/2017 in Central Ethiopia found the~~Earlier, the prevalence of *E. coli* was reported as~~of~~ 32.5% in backyard chicken while in this study higher number of *E. coli* of 51.11% were~~as~~ reported (17). ~~Another~~ Likewise, ~~research in Jordan (2016) found~~ the prevalence of 53.4% *E. coli* ~~of 53.4%~~ among broilers have been reported (18) while in our study ~~we got~~ 38.18 % prevalence of *E. coli* was seen among broilers (18). The difference in prevalence of *E. coli* infection in chicken might be due to the difference in geographical condition and climate.

Our study found the prevalence of *Salmonella* spp. to be 26 (12.26%) which three times lower than the prevalence reported in Egypt (54.4%) (19). Salmonellosis causes high mortality in

chicken and high economic loss to farmers (19). Any contamination of *Salmonella* spp. in human food may causes serious food borne infection (19). In this study, the prevalence of *Pseudomonas* s~~S~~pp. and *Pasteurella* s~~S~~pp. were 5.2% and 4.7% respectively. One Study ~~conducted in Egypt~~ found the low prevalence of *Pseudomonas* spp. of 2.2% (20). *Pseudomonas* spp. is distributed ubiquitous in nature (21). Infection of *Pseudomonas* spp. in chicken is caused from contaminated vaccines, needles of injection and wounds (21).

In our study, gentamicin and amikacin was found to most effective in majority of bacterial infection. Our study found high sensitivity of gentamicin followed by amikacin in *E. coli* and *Pseudomonas* spp. In *Salmonella* spp., amikacin was found to be effective followed by gentamicin. Furthermore, levofloxacin and doxycycline were found to be ineffective in majority of bacterial species isolated from chicken. Similarly, a study ~~done in Bangladesh~~ found gentamicin as effective antibiotic~~s~~ for treatment of infection caused by *E. coli* (22). In our study, 73.3% of E. coli were susceptible to gentamicin and similar pattern of about 60% of the *E. coli* isolated were earlier reported to be sensitive to gentamicin ~~in study done in Bangladesh~~ (22). Another study conducted by Thapa and Chapagain in Chitwan district found that amikacin was sensitive to 88.35% of *E. coli* (23). Our study recommends use of gentamicin or amikacin for treatment of bacterial infection in chicken in Chitwan district.

Our study found that 48.5% of *E. coli* were multidrug resistan~~ce~~. A study conducted in Chitwan, Nepal found that 96.12% of total isolated *E. coli* from diseased chicken were MDR (23). Multidrug resistance is emerging problem worldwide (4). ~~A study done in Bangladesh by~~ Sarkar et al (2019), Bashar et al (2011), Akond et al (2009) found that 100% isolates of *E. coli* were multidrug resistance (22, 24, 25). Our study demonstrate~~d~~ the prevalence of MDR *Pasteurella* s~~S~~pp., *Pseudomonas* s~~S~~pp., *Salmonella* s~~S~~pp., ~~were~~ as 50%, 18.2% and 13.9 % respectively.

Overall, our study found high prevalence of MDR bacteria among gram negative bacteria. The prevalence of MDR bacteria in our study was 42.5%. ~~A study conducted in China found~~Earlier high prevalence (88.2%) of MDR bacteria in chicken have been reported (26). Each year 700000 death are estimated to due antibiotic~~s~~ resistance and is expected to be increased by 10 million in year 2050 (27). Gram negative bacteria can acquire antibiotic genes through different antibiotic resistance mechanism (27). Under pressure of antibiotics, gram negative bacteria can undergo DNA mutation and can become antibiotic resistance (27). Another mechanism is that gram negative bacteria can also acquire antibiotic resistance gene from other bacteria present near to it through horizontal gene transfer (27).

Conclusion

This study showed high prevalence of m~~M~~ultidrug resistant~~ce~~ gram negative bacteria among different chicken breeds. This increase in multidrug resistant~~ce~~ bacteria have increased mortality rate in chicken, increased antibiotic use, decreased productivity, and increased the cost of production. In Nepal, routine microbiology test is not performed for detection and antibiotic susceptibility ~~test~~ for chicken pathogens. Veterinarians should prescribe antibiotics after performing antibiotic susceptibility test. To control the infection, f~~F~~armers should be aware on proper use of disinfectants in farm before adding new chickens.

Limitations

This study determines the prevalence of bacteria and multidrug resistant~~ce~~ bacteria in diseased chicken. Further study should focus on detection of metallo-beta-lactamase, extended spectrum of beta lactamase enzyme producing bacteria from chicken tissues.

List of Abbreviations

SIM: sulfide, indole, motility; MRVP: methyl-red, Voges Proskauer; MDR: multi-drug-resistant.

Declarations

Consent for publication

Not applicable.

Availability of data and material

All data obtained during this study are available within the article.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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