JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 273-282

Prevalence and Antimicrobial Resistance Characteristics of Gram-negative Bacteria Associated with Wild Animals Presenting at Live Animal Market, Taif, Western Saudi Arabia

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(Received: 10 August 2014; accepted: 31 October 2014)

Wildlife traded has the potential to carry or transmit potentially pathogenic and high resistant bacteria, that could have an important impact on human health. In this study, Gram-negative bacteria, associated antibiotic resistance and the presence of extended-spectrum beta lactamase (ESBL/AmpC) producing organisms in wild animals presenting at live animal market were investigated. Results revealed that 154 Gram-negative bacteria belong to eleven genera were recovered. Multiple drug resistance phenotypes were exhibited by 24% of tested isolates, the most common being against ampicillin, cephalothin, sulfonamide, aminoglycosides, tetracycline, and quinolones. Maximum resistance to extended-spectrum beta-lactam antibiotics occurred in 14.9% of isolates. The presence of ESBL/AmpC phenotype was detected in 11.0% of isolates as confirmed by combination disk method (CDM). The entire positive ESBL/AmpC producers were subjected to plasmid curing to ascertain the location of resistant marker. The result of the plasmid curing indicated that the resistance was chromosomally borne. Molecular analysis identified class 1 and 2 integrons among ESBL positive isolates. The findings have therefore established the presence of multidrug resistant bacteria and ESBL/AmpC producing organisms along with antibiotic resistant determinants in the fecal samples from wild animals and highlights the potential spread of pathogens and resistance between wild animals, environment and human.

Key words: Antibiotic resistance; Zoonoses, ESBL/AmpC; Plasmid curing; Integron; faecal bacteria; wildlife trade.

Wild animals are caught or bought for pet, shops, local breeder or traded (sometimes illegally). Wildlife harvest and trade range in magnitude from local communities hunting or gathering species for subsistence living to large commercial enterprises[1]. These live markets and the fact of animals and humans living so close together are known factors in the development of disease and also made it easier for organisms to spread, and often with negative implications for the environment and public health[2-3]. Furthermore, wild caught animals also may has the potential to introduce and spread bacteria which could potentially be resistant to antibiotics. The occurrence of antimicrobial resistance is important not only for the species studied but also for public health[4-7]. Recent studies indicate exponential increase in the frequency of antimicrobial resistance in clinical health settings[8]. Data from the Arabian Peninsula, including Saudi Arabia,

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suggested that multidrug resistant bacteria constitute a major problem in nosocomial and community-acquired infections[9-12]. A high prevalence of resistant phenotypes and genes had recently been reported in human[13-16], poultry[17], meat[18] and fresh vegetables[19] bacterial isolates from Saudi Arabia however, data quantifying the prevalence of antimicrobial resistant bacteria in wild caught traded animals are limited. The objective of this study was to evaluate the prevalence of enteric bacterial pathogens in the intestinal microbiota of pet wild animals presenting at live animal market Taif, Western Saudi Arabia. The study further aimed to determine the characteristics of antibiotic resistant and extended spectrum β-lactamase (ESBL) in gram negative bacilli isolates cultured.

MATERIALS AND METHODS

Sample collection

A total of 75 fecal samples were randomly taken from different wild mammals, birds, and reptiles presenting at live animal market, Taif, Western Saudi Arabia (Table 1). Approximately 1 g of fresh feces was collected, immediately placed in a sterilized containers with buffered peptone water (Oxoid), and transported under cooling condition to the laboratory where bacterial isolation was performed within 24 h.

Bacteriological analysis

Each sample was streaked into different bacteriological media such as Hektoen enteric agar (HE, Scharlau) and eosin methylene blue agar (EMB, Scharlau). Isolates were identified and confirmed using oxidase, catalase, and KOH and by commercially available biochemical test (API tests; bioMe'rieux). The API strips were analyzed according to the manufacturer's instructions. Reactions were recorded and identified using API Lab plus software (bioMe'rieux).

Antibiotic susceptibility profiles

The antibiotic resistance behavior of the isolated strains was determined on cation- adjusted Mueller-Hinton agar (Hi- Media) using Kirby-Bauer disk diffusion method according to the standards and interpretive criteria described by CLSI[20]. The antibiotics included were ampicillin (AMP 25), cephalothin (CEF 30), cefuroxime (CXM 30), cefotaxime (CTX 30) and ceftazidime (CAZ 30). Other antibiotics included cefepime (CEP, 30), aztreonam (AZT 30), and cefoxitin (FOX 30). β-lactam/ β-lactamase inhibitor combinations included amoxicillin/clavulanic acid (AMC 20/10). Imipenem (IMP 30) was used to test susceptibility to carbapenems. Non-β-lactam antibiotics included ciprofloxacin (CIP 10), sulfamethoxazolle/ trimethoprim (SXT 23.75/1.25), gentamycin (GEN 30), streptomycin (STR 10), tetracycline (TET 30) and chloramphenicol (CHL 30). All disks were purchased from Hi-Media and Mast-Diagnostics (Mast Group Ltd.) except for tetracycline and sulfamethoxazol-trimethoprim, which were purchased from Bioanalyse Ltd., and the results were recorded on the bases of the zone size interpretative chart supplied by the manufacturers. The quality control was performed to check the quality of medium and potency of antibiotic disks before use against some sensitive ATCC reference strains, including *E. coli* ATCC 25922 (beta-lactamase negative), *E. coli* ATCC 35218 (beta-lactamase positive), and *P. aeruginosa* ATCC 27853 (MicroTrol Discs; BD Diagnostics).

Phenotypic Detection of ESBL

Isolates which showed resistance to monobactam and cephalosporins, were screened for ESBL production by combination disk method (CDM) using cefotaxime and ceftazidime with and without clavulanic acid (Mast Diagnostics) according to Clinical and Laboratory Standards Institute (CLSI)[20]. Furthermore, a cefoxitin disk (30 mg, Mast Diagnostics) was added to this test, to detect AmpC phenotypes. All isolates classified as intermediate or resistant using CLSI criteria (≤17 mm) to cefoxitin were suspected to be AmpC. ESBL as well as AmpC phenotype were confirmed by MASTDISCSTM ID AmpC and ESBL test (Mast Diagnostics) and were subjected to further characterization.

Plasmid Curing

The ESBL producing isolates were selected and subjected to Sub-inhibitory concentration of sodium dodecyl sulfate (SDS) (Merck) mediated plasmid elimination[21]. The isolates were grown at 37°C for 24 hours in nutrient broth containing 10% SDS for plasmid curing. After which, the broth was agitated to homogenize the content and a loopful subcultured onto Mueller Hinton agar (MHA) plates and re-tested for ESBL production using combination disk method (CDM).

The curing was confirmed by loss of plasmid and cured markers were determined by comparison between the pre- and post- curing antibiograms of ESBL isolates. Loss of resistance markers gave an indication that those markers were probably located on a plasmid and not on the chromosome.

DNA extraction and amplification of class 1 and 2 integrons

Total DNA was obtained using the heat-shock technique. An overnight bacterial culture (200 μl) was mixed with 800 μl of distilled water, boiled for 10 min and, after cooling, centrifuged and the supernatant was used as the DNA template for the PCR. The detection of class 1 and class 2 integrons, PCR was performed with primers as previously described[22]. The PCR was conducted in a Thermal Cycler PXE-0.5 (THERMO; Electron Corporation) and the amplified product (10 µL) was electrophoresed on a 1.5% agarose gel in Tris-Borate-EDTA buffer at 80 V. The gels were stained with ethidium bromide and bands were visualized under a ultraviolet transluminator and photographed. The sizes of the amplified products were compared with 100 bp DNA mass marker.

RESULTS

Prevalence of Gram-negative bacteria isolated from wild animals

Table 2 summarizes the identification of the *Enterobacteriaceae* isolates retrieved. A total of 154 gram negative bacteria belong to 18 bacterial species in 11 genera were isolated from different wild mammals, reptiles and birds presented at live animal exhibit in Taif, Western Saudi Arabia. In many samples more than one species was isolated. The biochemical identification showed that predominant isolates in descending order of

Table 1. List of wild animals sampled at live animal market in Taif, Saudi Arabia

Animal	Scientific name	Number sampled
Rock Hyrax	*Procavia capensis*	5
Baboon Monkey	*Papio hamadryas*	1
Caracal	*Caracal caracal*	2
Common Kestrel	*Falco tinnuculus*	3
Peacock	*Pavo cristatus*	2
Common Quail	*Coturnix coturinx*	3
Laughing dove	*Streptopelia senegalensis*	1
Golden Hamaster	*Mesocricetus auratus*	2
Canaries	*Serinus canaria*	5
Arabian Hare	*Lepus capensis*	3
Arabian red fox	*Vulpes vulpes*	3
Lichtenstein's Sand grouse	*Pterocles lichtensteinii*	1
Chestnut-belled sand grouse	Pterocles *exustus*	1
Rock dove	*Columba livia*	3
Orange-winged parrot	*Amazona amazonica*	1
Long-tailed finch	*Taeniopygia guttata*	3
African grey parrot	*Psittacus erithacus*	1
Rose-ringed parakeet	*Psittacula krameri*	1
Tree pipit	*Anthus trivialis*	3
Yemen linnet	*Carduelis yemenensis*	4
Common myna	*Acridotheres tristis*	1
Albin Burmese python	*Python bivittatus*	1
Ball python	*Python regius*	1
Green Iguana	*Iguana iguana*	1
Hill Mynah	*Gracula religosa*	1
Common Mynah	*Acridotheres tristis*	1

frequency were *E. coli* (67 isolates; 43.5%), *Enterobacter aerogenes* (13 isolates; 8.4%), *Proteus mirabilis* (12 isolates; 7.8%), *K. pneumonia* (11 isolates; 7.1%), *Enterobacter cloacae* (9 isolates; 5.8%), *Klebsiella oxytoga* (7 isolates; 4.5%), *Pseudomonas aeruginosa* (6 isolates; 3.9%), *Citrobacter youngae* (5 isolates; 3.2%), 4 isolates (2.6%) of *Citrobacter freundii*, *Pseudomonas stuzeri*, three isolates (1.9%) of *Salmonella* spp., *Proteus vulgaris*, *Providencia rettgeri*, two isolates (1.3%) of *Morganella morganii*, *Citrobacter diversus*, and a single isolate (0.6%) of *Hafni alvei*, *Serratia oderferi*, *Escherichia vulneris*.

Phenotypic resistance and Incidence of multidrug resistant Gram-negative bacteria

The antimicrobial sensitivity phenotypes of recovered bacteria were determined by using a disk diffusion assay. The results of antibiotic sensitivity test were interpreted and are presented as the resistance of bacterial isolates to antibiotics (Table 3), and multiple antibiotic resistance among bacterial isolates (Table 4). The results showed that 37 isolates (24.0%) showed resistance phenotypes to three or more antimicrobial agents. The most common resistant species were *Escherichia coli* 17 (11.0%), and three isolates (1.9%) of *Klebsiella pneumonia* and *Klebsiella oxytoga*, respectively (Table 4). Apart from resistance to ampicillin and cephalothin antibiotics, a high rate of resistance was observed to sulfamethoxazole-trimethoprime (33.1%), tetracycline (27.9%), aminoglycosides (27.3%), cephalosporin's (14.9%), and quinolones (7.8%).

Phenotypic screening for ESBL isolates

Among the total of 154 tested isolates of Gram-negative bacteria derived from wild animals presented at live animal exhibit in Saudi Arabia, 23 (14.9%) ²-Lactam resistant isolates were obtained. That is, 20 isolates displayed ESBL phenotypes, and 3 isolates displayed AmpC phenotypes. The ESBL positive isolates were detected in 11.0% (n = 17) of the isolates. The most frequent ESBL-producing organism was *E. coli* (n = 9) (Table 5**)**.

Of note, the ESBL-producing strains were susceptible to carbapenems (100%) meanwhile resistance to cefepime was exhibited by 1.9% (3/154) strains. Resistance to β-lactamase inhibitors was observed in 5.7% (6/154) of the isolates.

Plasmid curing

The drug resistant marker of an organism could be chromosomal or extra chromosomal. The sensitivity is measured as the inhibition Zone Diameter (IZD) in millimetres. A close observation would reveal that the majority of isolates that tested positive for ESBL were resistant to CAZ and CTX after treatment with SDS. This result concluded that the resistance marker is chromosomally borne.

Antimicrobial resistance determinants

PCR screening detected class 1 integron in 4 isolates and class 2 integron could be detected in a single isolates of *P. mirabilis*.

DISCUSSION

This study describes prevalence and resistance to antimicrobial agents within Gram negative isolates from wild animals presented at live animal market in Taif, Western Saudi Arabia.

In this study, many Gram-negative bacterial isolates were recovered. The bacterial species isolated were *E. coli*, *K. pneumoniae*, *E. cloacae*, *E. aerogenes*, *K. oxytoca*, *C. freundii*, *C. youngae*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, and *Salmonella* spp. Similar gram negative bacterial

Table 2. Frequency of gram-negative bacteria isolated from fecal samples of wild animals at wild animal exhibit, Taif, Western Saudi Arabia

Bacterial isolates	*N*	Percentage
Escherichia coli	67	43.5
Proteus mirabilis	12	7.8
Klebsiella pneumineae	11	7.1
Enterobacter cloacae	9	5.8
Pseudomonas aeruginosa	6	3.9
Enterobacter aerogenes	13	8.4
Citrobacter freundii	4	2.6
Morganella morganii	2	1.3
Citrobacter youngae	5	3.2
Klebsiella oxytoca	7	4.5
Pseudomonas stuzeri	4	2.6
Providencia rettegrii	3	1.9
Proteus vulgaris	3	1.9
Citrobacter diversus	2	1.3
Salmonella spp.	3	1.9
Hafnia alvei	1	0.6
Serratia odorifera	1	0.6
Escherichia vulneris	1	0.6
Total	154	100

Table 3. Antibiotic resistance phenotype in Gram-negative bacterial population of wild animals presented at wild animal market, Taif, Saudi Arabia

Bacterial species (n)	*Beta-lactams& inhibitors*									*Amino-glycosides*		*Quino lone*	*Macro lide*	*Sulfon amide*	*Phen icole*	*Tetra cycline*
	AMC	AMP	CEF	ATZ	CXM	CAZ	CTX	CEP	FOX	GEN	STR	CIP	ERY	SXT	CHL	TET
E. coli (67)	2	51	29	8	7	5	6	1	1	3	16	7	3	25	4	15
K. pneuminea (11)	0	8	7	1	2	1	1	0	0	1	5	1	0	3	1	4
P. mirabilis (12)	0	9	5	1	2	1	1	0	0	1	3	1	1	2	1	2
E. cloacae (9)	1	5	4	1	1	0	1	0	0	1	3	0	1	3	0	2
Ps. aeruginosa (6)	0	0	1	0	0	0	0	0	0	0	3	0	0	3	0	1
E. aerogenes (13)	0	7	3	1	1	0	1	0	0	1	0	0	0	1	0	0
K. oxytoca (7)	2	4	3	1	1	1	1	1	1	0	0	1	1	2	1	3
M. morganii (2)	0	1	1	1	1	0	1	0	0	0	1	0	0	1	0	1
C. youngae (5)	1	3	2	1	1	1	1	0	0	0	2	0	1	2	0	3
C. freundii (4)	0	3	2	1	1	1	1	1	0	0	2	1	1	1	1	3
C. diversus (2)	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
P. vulgaris (3)	0	1	1	1	1	0	1	0	0	1	1	0	0	2	1	1
S. odorifera (1)	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1
H. alvei (1)	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1
Ps. stuzeri (4)	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1
P. rettegarii (3)	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1
E. vulnaris (1)	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Salmonella spp. (3)	0	1	1	1	1	0	1	0	0	0	2	1	0	1	0	3
Overall, *n* = 154 (%)	6 (3.9)	96 (62.3)	61 (39.6)	19 (12..3)	19 (12.3)	10 (6.5)	16 (10.4)	3 (1.9)	2 (1.3)	8 (5.2)	42 (29.2)	12 (7.8)	9 (5.8)	51 (33.1)	9 (5.8)	43 (27.9)

AMC, amoxicillin–clavulanic acid; AMP, ampicillin; AZT, aztreonam; CAZ, ceftazidime; CEF, cephalexin; CEP, cefepime; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; CXM, cefuroxime; FOX, cefoxitin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole–trimethoprim; TET, tetracycline.

species were also isolated from wild animals by other researchers[6,23]. Liu *et al*.,[24] identified six different Gram-negative genera from zoo captured and free-ranging turtles. Recently, Shobrak *et al*.,[25] identified a variety of gram negative bacteria associated with captive houbara bustard reared national wildlife research center, Taif, Saudi Arabia. Determining microbial antibiotic resistance from wild animals is an important finding. Our results show that wild animals presented at live animal market, Taif, Western Saudi Arabia are more likely to shed antibiotic resistant bacteria, and their faeces are more likely to harbor multidrug resistant bacteria. The higher prevalence of antibiotic resistance and multiple antibiotic resistance in this study fit well with previous observations in wild animals [6, 25, 26, 27, 28]. Early in Japan, Nakamura *et al*.[29] showed that pet birds imported from tropical countries were found to carry MDR bacteria. In the same environment of Western Saudi Arabia, Abulreesh[30] observed multidrug resistance among *E. coli* isolates from rock pigeon. All wild animals presented at live animal markers are coexisted besides domestic mammals and birds, and from private owners that may have antibiotics from treatment or prophylaxis. Thus, the higher antibiotic resistance could reflect the condition. Transportation and other stress factors including keeping in cages have also been shown to increase the shedding of antibiotic resistant enterobacteria[31]. It would seem that *E. coli* was the most common isolated bacteria from wild animals. Animals living in urbanized areas or with close contact with human are more likely to carry *E. coli* than animals living in remote areas[32, 33]. However, Guenther *et al*.[34] reported no significant difference in the isolation frequency of muliresistant *E. coli* strains between rural or urban areas.

Table 4. Incidence of MDR Gram-negative bacteria isolated from wild animals at wild animal exhibit in Taif, Western Saudi Arabia*

Bacteria	Number of MDR (%)
E. coli	17 (11.0)
K. pneumonia	3 (1.9)
P. mirabilis	2 (1.3)
E. cloacae	2 (1.3)
K. oxytoga	3 (1.9)
E. aerogenes	1 (0.6)
C. youngae	2 (1.3)
P. aeruginosa	1 (0.6)
M. morganii	1(0.6)
C. freundii	1(0.6)
P. vulgaris	1 (0.6)
S. odorifera	1 (0.6)
Salmonella spp.	2(1.3)
Total (%)	37 (24.0)

*MDR indicates resistance against more than 3 antimicrobial class.

Table 5. Prevalence of β-Lactamase among total and ESBL positive Gram-negative bacteria derived from wild animals presented at live animal exhibit in Taif, Western Saudi Arabia

Bacterial species	ESBL resistance No (%)	ESBL positive No (%)
E. coli	10 (6.5)	9 (90.0)
K. pneumonia	2 (1.3)	1 (50.0)
P. mirabilis	2 (1.1)	1 (50.0)
E. cloacae	1 (0.6)	1 (100)
K. oxytoga	1 (0.6)	1 (100)
C. youngae	1 (0.6)	1(100)
C. freundii	2 (1.3)	1 (50.0)
P. vulgaris	1 (0.6)	1 (100)
E. aerogenes	1 (0.6)	1 (100)
M. morganii	1(0.6)	-
Salmonella spp.	1 (1.3)	-
Overall n =154 (%)	23 (14.9)	17 (11.0)

Apart of ampicillin and cephalothin, high resistance rates and resistance phenotypes were recorded to sulfamethoxazole-trimethoprim, extended-spectrum beta-lactam, tetracycline streptomycin and ciprofloxacin. Similar resistance phenotypes have been previously recorded for strains of Gram negative bacteria and *E. coli* isolated from wild animals in Portugal[35], from free-living Canada geese in Georgia and North California[36], from black-headed gulls in the Czech Republic[37], from zoo animals in Japan[6], from parrots seized from illegal wildlife trade[23], and captive houbara bustard in United Arab Emirates[38] and Saudi Arabia[25].

The resistance to extended-spectrum beta-lactam and beta-lactamase inhibitors is of great clinical significance in several countries. Resistance to beta-lactam antibiotics is primarily mediated by beta-lactamases production. In this study, maximum resistance rate to the extended-spectrum beta-lactam antibiotics was expressed by

14.9% of isolates, likewise 11.0% was shown to be ESBL producers. Previous reports have described ESBL-containing bacteria in healthy wild animals[34, 39]. Ahmed *et al*.[6] showed that may isolates of gram-negative were resistant to ESBL antibiotics. The detection rates of ESBL-*E. coli*, the head of large bacterial family, *Enterobacteriacae*, in different geographical areas ranged from 0.5% in birds of the remote Azores islands in the Atlantic Ocean[40] to 32% for birds of the Iberean peninsula[41]. Others found ESBL-producing *Enterobacteriaceae* in up to 59% of the crows in Bangladesh[42]. A lower prevalence of ESBL-*E. coli* was also observed (0.8%) in glaucous-winged gulls of Kamchatka peninsula in Russia[43]. None of the tested isolates in this study were resistant to carbapenems. In line with this result, Hasan *et al*.[7] stated that screening for carbapenemase producer among *E. coli* in wild birds and free-range poultry yield no isolates. While, Ahmed *et al*.[6] detected resistance to imipenem in gram-negative bacteria recovered from zoo wild animals in Japan.

The drug resistant of an organism could be chromosomal or extra chromosomal[44]. The results of plasmid curing concluded that the resistance marker is mostly. Similarly, Ikegbunam *et al*.[45] indicated that the plasmid curing of ESBL-positive isolates from animals at slaughter were chromosomally borne. Others in his study, Enabulele *et al*.[46] reported that the resistance of Gram negative bacteria isolated from infected wounds from the university teaching hospital was plasmid mediated, meanwhile, in a study carried out by Yah *et al*.[21] indicated that resistance of Gram negative bacteria from hospital sources were plasmid mediated and chromosomal mediated resistance.

Regarding characterization of ESBL-positive isolates for presence of integrons. PCR screening detected class 1 integron in 4 isolates and class 2 integrone could be detected in a single isolates of *P. mirabilis*. Detection of class 1 and 2 integrons have been previously identified from *E. coli* free-living geese[36], Zoo animals[6], and gulls[37]. Furthermore, class 1 integrons and associated resistance genes were detected in 48% of fecal samples from captive wallabies, however, no integrons were detected in animals from five wild populations[47].

A comparative view on the resistance patterns of the bacterial species as well as ²-lactamase phenotypes described in our study with those of bacterial species originating from clinical settings in Saudi Arabia and Arabic Gulf region revealed a correlation. In Saudi Arabia, *E. coli* isolated from chicken intestine was found to be resistant to many antibiotics[17]. In line with this result, Altalhi *et al*.[18] observed high rates of resistance among *E. coli* isolates from chicken meat against sulfonamides, nalidixic acid, gentamicin, chloramphenicol, and streptomycin. Further, a gradual increase in resistance rates against fluoroquinolones, aminoglycosides, and trimethoprime- sulfamethoxazole and multidrug resistance among bacteria had been reported in many retrospective analysis[13-16]. In recent years, data from Arabian Gulf region showed high occurrence of ESBL-producing isolates, with rates as high as 41% in United Arab Emirates[10], 31.7% in Kuwait[11], and 55% in Saudi Arabia[12].

In summary, the present data induce deep concerns on the dissemination of resistance to antimicrobial agents and highlight traded wild animals as a potential sources of resistant bacteria and clinical important resistance genes.

ACKNOWLEDGEMENTS

Sincere thanks to Mr. Abo-bakre Al-Aedarouse for technical assistance and Mr. Samy El-Sewat for help in sample collections. This work was supported by grant from the Taif University (project 1/433/1891).

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