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Isolation and Detection of Methicillin-Resistant Staphylococci in Healthy Broilers in Nsukka Southeast, Nigeria

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Abstract

This study was conducted to isolate and detect methicillin-resistant staphylococci (MRS) in healthy broilers in Nsukka Southeast, Nigeria and determine the antibiogram of the isolates. Cloacal and skin swabs were collected from each of 101 randomly sampled broilers meant for slaughter. The samples were processed for isolation and identification of methicillin-resistant *Staphylococcus* species, following standard methods. Confirmation of methicillin-resistance by the isolates was done using penicillin binding protein 2a (PBP2a) kit. Phenotypic resistance of the isolates to antimicrobial agents was determined using disc diffusion method. Out of 202 samples processed, 200 (99.01%) yielded positive growth of staphylococci on oxacillin-supplemented oxacillin-resistance staphylococcal agar base (ORSAB). A total of 200 methicillin-resistant staphylococcal isolates were obtained. Of these, 91 (45.5%) were identified as methicillin-resistant coagulase-positive *Staphylococcus* (MRCoPS), while 109 (54.5%) were identified as methicillin-resistant coagulase-negative *Staphylococcus* species (MRCoNS). Out of the 91 MRCoPS, 53 (58.2%) were identified as methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance of the isolates was 99.5% to erythromycin and chloramphenicol, 100% to oxacillin, 76.5% to gentamicin, 96.5% to clindamycin, 92.5% to ciprofloxacin, 99% to sulphamethoxazole/trimethoprim and tetracycline, and 98.5% to streptomycin and cefoxitin. All the isolates were multidrug resistant. This study has shown that healthy broilers reared and slaughtered in Nsukka Southeast, Nigeria harbour multidrug-resistant MRS and thus serve as their reservoirs.

Keywords: gallinaceous, staphylococcus, multidrug-resistance

Introduction

The increased use of antimicrobial agents in both human and veterinary medicine has led to the emergence of antimicrobial resistance in bacterial organisms (Geser *et al*., 2012). In food-producing industry, for animals, especially poultry, different antimicrobial agents are used extensively in sub-therapeutic/therapeutic doses for growth promotion, routine disease prevention and treatment of bacterial diseases (Gilchrist *et al*., 2007; Waters *et al*., 2011). This indiscriminate practice is usually worse in developing countries including Nigeria, where there are no strict regulations on the use of antimicrobials in food-producing animals. This has led to increased resistance to most of the antimicrobials used in food animal production, especially the beta-lactams (penicillins) and cephalosporins (Wulf and Voss, 2008). Staphylococci are opportunistic pathogens, part of normal commensal flora of skin and mucous membranes of food-producing animals (Quinn and Markey, 2003). They are among the most prevalent causes of clinical infections globally and have garnered substantial public attention due to increasing mortality associated with antimicrobial resistance (Waters *et al*., 2011). Staphylococci resist all beta-lactams by the production of beta-lactamases (Livermore and Brown, 2001; Quinn and Markey, 2003). The use of beta-lactamase-resistant cephalosporins (such as methicillin or oxacillin) as a solution to beta-lactam resistance in staphylococci was short-lived when these organisms also became resistant to the beta-lactamase-resistant cephalosporins (Wulf and Voss, 2008). Staphylococcal isolates that are resistant to methicillin are those that have acquired *mec A* gene located on staphylococcal chromosomal cassettes (SCCs), which encodes the expression of penicillin binding protein 2a (PBP2a), a factor that reduces the binding affinity of penicillins and cephalosporins to the cell wall of organisms (Weese and Van Djuikeren, 2010).

Isolation of methicillin-resistant staphylococci (MRS) from livestock generated a lot of interest in recent years (Wulf and Voss, 2008; Febler *et al*., 2011). Identification of livestock-associated MRS (LA-MRS) in food-producing animals raised questions regarding the presence of MRS in food of animal origin and its impact on food chain (Klutymans, 2010; Kwon *et al*., 2006; Febler *et al*., 2011). Epidemiological studies showed that LA-MRS not only colonized livestock which served as their reservoirs, but also contaminated meat of both colonized animals and that of

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others during slaughter, overcame species barrier and resulted in zoonotic infections in humans with direct (handling and consumption of contaminated meat) and indirect exposure to the livestock and their products (Ekkelenkamp *et al*., 2006; Fitzgerald, 2012; Schaumburg *et al*., 2013). LA-MRS are considered major pathogens causing nosocomial and many hospital-linked infections worldwide (Febler *et al*., 2011). Outcomes of these infections are often fatal because of the complications and difficulty of their treatment, which is associated with multidrug resistance of LA-MRS (Febler *et al*., 2011). Therefore, determination of antimicrobial resistance profile of LA-MRS isolates is crucial for empirical treatment of infections associated with LA-MRS.

Recently, there is an increasing numbers of reports on the isolation of LA-MRS from humans, farm and slaughterhouse environments and animals (Mulders *et al*., 2010; Petinaki and Spiliopoulous, 2012). This necessitated surveillance studies to assess food-producing animals as potential reservoirs of MRS in different parts of the world such as America (Smith *et al*., 2008; Waters *et al*., 2011), Europe (Armand-Lefevre *et al*., 2005; Friese *et al*., 2012; Huijsdens *et al*., 2006; Meemken *et al*., 2008; Pomba *et al*., 2009; Voss *et al*., 2005;), Asia (Lee, 2003; Moon *et al*., 2007) and South Africa (Ateba *et al*., 2010). These studies were conducted in food-producing mammals. Surveillance studies to detect the occurrence of LA-MRS in poultry are rather scanty. However, studies conducted in Belgium (Nemati *et al*., 2008; Nemeghaire *et al*., 2013; Persoons *et al*., 2009), The Netherland (Mulders *et al*., 2010) and Germany (Friese *et al*., 2013; Schaumburg *et al*., 2013) implicated poultry as potential reservoir of MRS.

In available literature, there is only a report on surveillance of food-producing animals (ruminants) as reservoirs of MRS in Nigeria (Mai-siayama *et al*., 2014). No study has been conducted to screen poultry birds reared and slaughtered in Nsukka Southeast, Nigeria, as potential reservoirs of MRS, whereas broilers constitute a major source of protein for the Nsukka populace. These broilers may habour MRS which are consumed together with the broiler meat by the Nsukka population, hence increasing the dissemination of antimicrobial resistance genes. The objective of this study, therefore, was to isolate and detect MRS in broilers slaughtered in Nsukka Southeast, Nigeria and determine the antimicrobial resistance profile of the isolates.

Materials and methods

Sampling

Broilers meant for slaughter at Nsukka abattoir between May and August 2014 were sampled. A total of 101 broilers were randomly selected. Cloaca and breast skin swabs were collected from each of the bird using sterile swab sticks moistened with sterile normal saline. The samples were transported aseptically and processed within 1 hour of collection in the Veterinary Microbiology Laboratory, Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka.

Isolation of presumptive methicillin-resistant staphylococci from broilers

The swabs from cloaca and breast skin were aseptically inoculated into brain heart infusion broth supplemented with 6.5% sodium chloride for selective isolation of staphylococci and incubated at 37 °C for 24 hours aerobically. A loopful of the cultures was sub-cultured onto oxacillin-resistance staphylococcal agar base (ORSAB) supplemented with 1.0 mg of oxacillin and 25,000 IU of polymyxin, and incubated at 37 °C for 24 hours. The morphology of different colonial types were appropriately described and recorded. Purification of the cultures was done using nutrient agar supplemented with 6.5% sodium chloride and incubated at 37 °C for 24 hours. Pure cultures of the staphylococcal isolates were then inoculated onto slants of nutrient agar supplemented with 6.5% sodium chloride, incubated at 37 °C for 24 hours and stored in refrigerator at 4 °C as stock cultures until needed for further analysis. Phenotypic characterization of the isolates was done by subjecting them to various tests such as Gram staining, catalase, coagulase, haemolysis and biofilm following standard procedures.

Evaluation of PBP2a production by presumptive methicillin-resistant staphylococcal isolates from broilers

The staphylococcal isolates were assessed for the production of PBP2a using the Slidex MRS Detection, which is based on the agglutination of latex particles sensitized with monoclonal antibodies against PBP2a. The test was carried out and interpreted according to the manufacturer's instructions.

Determination of antibiogram of methicillin-resistant staphylococcal isolates from broilers

Antimicrobial susceptibility of the MRS isolates was determined by the disc diffusion method (CLSI, 2012). The isolates were sub-cultured on nutrient agar, incubated at 37 °C for 24 hours. Then colonies of each of the isolate were adjusted to 0.5 McFarland's turbidity standard (equivalent to 1×10^8 colony forming unit/ml) in sterile nutrient broth. The standardized broth cultures were incubated for 10 minutes at 37 °C and then inoculated onto sterile Mueller-Hinton agar plates using sterile swab stick. Ten antibacterial agents were used and they included: oxacillin (1 μg), erythromycin (15 μg), choramphenicol (30 μg), streptomycin (10 μg), tetracycline (30 μg), gentamicin (30 μg), clindamycin (2 μg), ciprofloxacin (5 μg), sulphamethoxazole/trimethoprim (25 μg) and cefoxitin (30 μg). The discs were placed strategically on the inoculated nutrient agar plate. The plates were incubated at 37 °C for 24 hours. After incubation the zone of inhibition around each disc was measured with a meter rule. Each test was performed in triplicate and the mean inhibitory zone diameter (IZD) calculated to the nearest whole millimeters for each isolate and each antibacterial agent. The IZD was interpreted as susceptible, intermediate or resistant according to the Clinical and Laboratory Standards Institute (CLSI) (2012) criteria for aerobic isolates.

Results

Isolation rates of MRS from broilers

Out of 202 samples processed, 200 (99.01%) yielded positive growth of staphylococci on oxacillin-supplemented ORSAB (Table 1). From these positive samples, a total of 200 presumptive methicillin-resistant staphylococcal (pMRS) isolates were obtained. All the pMRS isolates were positive for PBP2a production test. Out of these 200 methicillin-resistant

staphylococcal isolates, 91 (45.5%) were identified as coagulase-positive *Staphylococcus* (MRCoPS), while 109 (54.5%) were identified as coagulase-negative *Staphylococcus* species (CoNS) (Table 2). Out of the 91 MRCoPS, 53 (58.2%) were obtained from cloaca samples, while 38 (41.8%) were obtained from the breast skin samples. Out of 91 MRCoPS, 53 (58.2%) were identified as methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Of these, 33 (62.3%) isolates were obtained from the cloaca samples, while 20 (37.7%) isolates were obtained from the breast skin samples. Of the 109 MRCoNS, 47 (43.1%) were isolated from cloaca samples, while 62 (56.9%) were isolated from breast skin samples.

Table 1. Isolation rate of methicillin-resistant staphylococci from broilers

Sample	Number of broilers sampled	Number of positive culture	Number (Percentage) of isolates obtained	Number of isolates positive for PBP2a
Cloaca swab	101	100	100 (99.01)	100
Breast skin swab	101	100	100 (99.01)	100
Total	202	200	200	200

PBP2a = Penicillin binding protein 2a

Table 2. Isolation rates of methicillin-resistant *Staphylococcus* species from broilers

Sample	Number of isolates obtained	Number (Percentage) of isolates obtained		
		MRCoPS	MRSA	MRCoNS
Cloaca swab	100	53 (58.2)	33	47 (43.1)
Breast skin swab	100	38 (41.8)	20	62 (56.9)
Total	200	91 (45.5)	53 (58.2)	109 (54.5)

MRCoPS = methicillin-resistant coagulase-positive *Staphylococcus*, MRCoNS = methicillin-resistant coagulase-negative *Staphylococcus* species, MRSA = methicillin-resistant *Staphylococcus aureus*

Antibiogram of methicillin-resistant staphylococcal isolates from broilers

Out of 200 isolates, 199 (99.5%) isolates were resistant to erythromycin, 200 (100%) to oxacillin, 153 (76.5%) to gentamicin, 199 (99.5%) to chloramphenicol, 193 (96.5%) to clindamycin, 185 (92.5%) to ciprofloxacin, 198 (99%) to sulphamethoxazole/trimethoprim, 198 (99%) to tetracycline, 197 (98.5%) to streptomycin and 197 (98.5%) to cefoxitin (Table 3 and Fig. 1). The methicillin-resistant staphylococcal isolates exhibited 14 multidrug resistance patterns with E-OX-GN-C-DA-CIP-SXT-TE-S-FOX being the most prevalent pattern (Table 4). Out of 200 isolates, 1 (0.5%) was resistant to 2 and 5 classes of the antibacterial agents tested, 3 (1.5%) were resistant to 6 classes, 14 (7%) to 7 classes and 181 (90.5%) to 8 classes (Table 5).

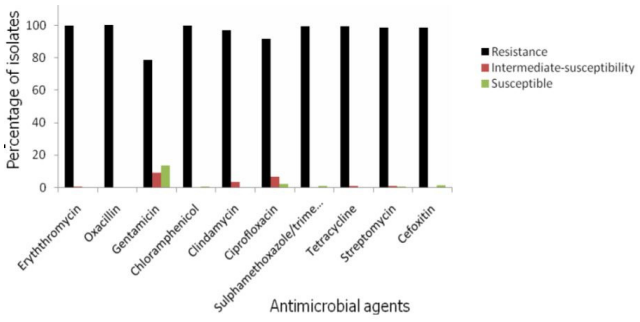

Fig. 1. Antibiogram of methicillin (oxacillin) resistant staphylococcal isolates from broilers

Table 3. Antibiogram of methicillin-resistant staphylococcal isolates from broilers

Antimicrobial agent	Potency (μg)	Number (Percentage) of isolate (*n= 200*)		
		Resistant	Intermediate	Susceptible
Erythromycin	15	199(99.5)	1(0.5)	0(0)
Oxacillin	1	200(100)	0(0	0(0)
Gentamicin	30	153(76.5)	19(9.5)	28(14)
Chloramphenicol	30	199(99.5)	0(0)	1(0.5)
Clindamycin	2	193(96.5)	7(3.5)	0(0)
Ciprofloxacin	5	185(92.5)	12(6)	3(1.5)
Sulphamethoxazole/ trimethoprim	25	198(99)	0(0)	2(1)
Tetracycline	30	198(99)	2(1)	0(0)
Streptomycin	10	197(98.5)	2(1)	1(0.5)
Cefoxitin	30	197(98.5)	0(0)	3(1.5)

Table 4. Frequency of resistance patterns exhibited methicillin-resistant staphylococcal isolates from broilers

S/N	Resistance pattern	Frequency	Percentage
1	OX-DA-FOX	1	0.5
2	OX-C-CIP-TE-S-FOX	1	0.5
3	E-OX-C-DA-CIP-TE-S-FOX	1	0.5
4	E-OX-C-DA-SXT-TE-S	1	0.5
5	E-OX-C-DA-SXT-S-FOX	1	0.5
6	E-OX-C-SXT-TE-S-FOX	1	0.5
7	E-OX-C-DA-SXT-TE-S-FOX	2	1
8	E-OX-GN-C-CIP-SXT-S-FOX	1	0.5
9	E-OX-GN-C-DA-CIP-SXT-TE-S	1	0.5
10	E-OX-GN-C-DA-CIP-SXT-TE-FOX	1	0.5
11	E-OX-GN-C-DA-SXT-TE-S-FOX	7	0.35
12	E-OX-GN-C-CIP-SXT-TE-S-FOX	3	1.5
13	E-OX-C-DA-CIP-SXT-TE-S-FOX	41	20.5
14	E-OX-GN-C-DA-CIP-SXT-TE-S-FOX	138	69
	Total	200	100

E = erythromycin, OX = oxacillin, GN = Gentamicin, C = competentelor, DA = clindamycin, CIP = ciprofloxacin, SXT = sulphamethoxazole/timethoprim, TE = tetracycline, S = streptomycin, FOX = cefoxitin

Table 5. Number of antimicrobial class to which methicilin-resistant staphylococcal isolates from broilers were resistant

Number of antimicrobial class	Number (Percentage) of isolates resistant
2	1(0.5)
5	1(0.5)
8	181(90.5)
7	14(7)
6	3(1.5)
Total	200(100)

Discussions

In this study, MRS were isolated from cloaca and breast skin of broilers slaughtered in Nsukka Southeast, Nigeria and the antibiogram of the isolates was determined. The fact that all the isolates grew on oxacillin-supplemented ORSAB suggest that they are methicillin-resistant strains. Production

of PBP2a by all the isolates confirmed that they are methicillin-resistant strains and thus may harbour *mec A* gene which encodes for the expression of PBP2a (Peersons *et al*., 2009). Isolation of 200 (99.01%) staphylococcal isolates from 202 samples processed indicates that the medium used (oxacillin-supplemented ORSAB) was highly selective and effective for primary isolation of MRS from the samples. The 99.01% MRS isolation rate from both cloaca and breast skin of the sampled broilers, suggests that both sites were highly colonized by MRS. The high isolation rate of MRS in this study suggests that the farms in which these birds were raised could be heavily contaminated with MRS. Colonization of farm environments including poultry farms by MRS have been reported (Petersen *et al.,* 2013). The isolates could have colonized the breast skin of the broilers when they lied down on contaminated litter, since they use their breasts to make direct contact with the floor. It is also possible that the handlers of the birds transferred the organisms to the birds. Reports have shown that MRS can be transferred from humans, especially animal handlers, to animals on direct and/or indirect contact (Petinaki and Spiliopoulous, 2012). However, colonization of the cloacae of the birds may have resulted following ingestion of the MRS from contaminated litter. Staphyloccoci constitute part of the normal flora of the skin and mucous membrane of birds (Quinn and Markey, 2003), hence, it is possible that the isolates acquired methicillin-resistance genes from other bacterial organisms picked from their environment or those contracted from their handlers. The MRS isolation rate recorded in this study is higher than 34.6 and 41% MRS isolation rate reported by Mai-siayama *et al*. (2014) and Waters *et al*. (2011) from ruminants and processed chicken in Northeast Nigeria and America, respectively. Pletinckx *et al*. (2010) reported 44.4 and 16.7% MRSA isolation rate from claoca and skin beneath the wings of broilers, respectively. The variation in the isolation rates in these studies may be related to differences in the rate of contamination of the handlers, farm environments and the animals. The finding in this study suggests that broiler farms in the study area are heavily contaminated with MRS. This may imply that acquisition of methicillin-resistance genes is going on at a tremendous rate and proportion among staphylococci colonizing broilers reared and slaughtered in Nsukka. This finding portends health risks to the handlers of these broilers and the consumers.

In this study, the 53 (58.2%) isolates among the MRCoPS identified as MRSA, produced coagualse and haemolysin, which are important virulent factors of *Staphylococcus aureus* (Quinn and Markey, 2003). Production of coagulase enables the organism to evade the phagocytic cells while invading its host, whereas haemolysin destroys the host's erythrocytes releasing important nutrients such as iron for bacterial growth (Jarraud *et al*., 2002). However, the non-haemolytic MRCoPS isolates obtained in this study could be other coagualse-positive staphylococcal species such as *S. pseudintermedius*, *S. schlieferi*, *S. delphini*, *S. intermedius* that colonize farm animals (Morgan, 2008; Quinn and Markey, 2003; Wesse and van Djuikeren, 2010). The Isolation of 109 (54.5%) MRCoNS in this study suggests that high number of commensal CoNS associated with food-producing animals may be harbouring *mec A* gene. This finding is important because most investigators tend to neglect the role of CoNS as potential reservoirs and source of transfer of resistance genes among bacterial organisms. CoNS are usually saprophytes in farm environments, thus they are associated with food-producing animals (Morgan, 2008; Quinn and Markey, 2003). The MRCoNS isolation rate in this study is higher than 6.7% MRCoNS isolation rate reported by Nnachi *et al*. (2014) from raw meats (beef, pork, goat and donkey) marketed in Southeast, Nigeria.

The 100% oxacillin-resistance recorded further proves that the staphylococcal isolates are methicillin-resistant strains. The oxacillin-resistance rate is similar to 100% oxacillin-resistance reported by Gali *et al*. (2013) among methicillin-resistant staaphylococcal isolates from bovine milk in Northeast, Nigeria. High rates of resistance to cefoxitin (98.5%) and ciprofloxacin (92.5%) in this study suggests again that the isolates may be harbouring *mec A* gene. Resistance to cefoxitin is used for the confirmation of methicillin-resistance in bacterial organisms (Perry *et al*., 2004). Resistance to ciprofloxacin is used as a surrogate marker for the identification of MRS (Kunori *et al*., 2002). The ciprofloxacin resistance observed is higher than 35 and 80% ciprofloxacin resistance, reported by Mulders *et al*. (2006) and Waters *et al*. (2011) among MRSA isolates from broilers and poultry, and processed chicken in Belgium and America, respectively. Pearsoons *et al*. (2009) reported 100% ciprofloxacin resistance among MRSA isolates from poultry in Belgium, which is higher than the findings in this study.

High rates of resistance to erythromycin (99.5%), gentamicin (76.5%), chloramphenicol (99.5%), clindamycin (96.5%), streptomycin (98.5%), sulphamethoxazole/trimethoprim (99%) and tetracycline (99%) suggests that the isolates exerted selection and resistance to these drugs. High rate of streptomycin resistance recorded may be as a result of its frequent use in the study area. Streptomycin is often used with penicillins to exert a broad-spectrum action during treatment of infections in poultry. Resistance to streptomycin may have resulted to the high gentamicin resistance since a gene can encode for resistance to many aminoglycosides (Febler *et al*., 2011). The gentamicin resistance rate noted is higher than 14.8, 38 and 43.5% reported by Nemati *et al*. (2008) and Mulders *et al*. (2006), and Waters *et al*. (2011) among MRSA isolates from cloacae of farmed broilers and broilers meant for slaughter, and processed chicken in Belgium and America, respectively. Mulders *et al*. (2006) and Nemati *et al*. (2008), and Waters *et al*. (2011) reported 55, 37 and 8.7% erythromycin resistance rates among MRSA from broilers in Belgium and America, respectively, which is lower than the findings (99%) in this study. Chloramphenicol resistance is comparable to 100% chloramphenicol resistance reported by Pearsoons *et al*. (2009) among MRSA isolates from poultry in Belgium, but it is higher than the findings of Nemeghaire *et al*. (2013), who reported 40% chloramphenicol resistance among MRSA isolates from broilers and layers in Belgium.

The high tetracycline resistance obtained may be due to indiscriminate use of drugs in broiler production in the study area. Tetracycline is a broad-spectrum antibiotic frequently used for growth-promotion and treatment of bacterial infections in poultry, especially for broilers production in Nigeria (Oluwasile *et al*., 2014). Thus, the isolates may have been exposed to tetracycline resulting in development of resistance against it. The 99% tetracycline resistance in this

study is comparable to 90 and 96.9% tetracycline resistance reported by Mulders *et al*. (2006) and Febler *et al*. (2011) among MRSA isolates from broilers meant for slaughter and imported fresh poultry meat in Belgium and Germany, respectively. It is higher than 58.8% reported by Nemati *et al*. (2008) among MRSA isolates from broilers in Belgium. The clindamycin resistance rate contrasts that of Mulders *et al*. (2006) and Waters *et al*. (2011) who reported 54 and 8.7% clindamycin resistance among MRSA isolates from poultry meat in Belgium and America, respectively.

In terms of resistance to classes of antibacterial agents, 198 (99%) of the isolates were resistant to three or more classes of the antibacterial agents tested, thus indicating multidrug resistance. The resistance patterns exhibited by the isolates proved further that the isolates were resistant to three or more of the drugs tested. LA-MRS has been widely reported to be multidrug resistant (Waters *et al*., 2011). It is well documented that *mec A* gene, which encode for methicillin-resistance in staphylococci, also encodes for resistance to other classes of antimicrobial agents including aminoglycosides, lincosamides, sulfonamides and tetrayclines (Febler *et al*., 2011; Morris, 2006b). Therefore, the multidrug resistance exhibited by the isolates in this study, pose great health threat to the public (Nemati *et al*., 2008).

Conclusions

This study has shown that MRS are associated with broilers reared and slaughtered in Nsukka Southeast, Nigeria, thus these broilers are potential reservoirs for MRS. Since the isolates are multidrug-resistant, they portend adverse impact on the food chain. However, molecular characterization of the isolates to elucidate *mec A* gene and other genes encoding for resistance to the other antibacterial agents is recommended.

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