ANTIMICROBIAL RESISTANCE IN ENTEROCOCCI ISOLATED FROM POULTRY IN OWERRI, IMO STATE NIGERIA

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Abstract: Poultry are increasingly being associated with carriage of multi-resistant organisms that may cause disease in humans. This study evaluated the prevalence and antimicrobial resistance in enterococci isolated from poultry in Imo State Nigeria. One hundred and thirty (130) *Enterococcns* spp. were isolated from cloacal swabs, fecal and litter samples from different poultry farms. The isolates were characterized microbiologically and biochemically by fermentation tests. Theywere then evaluated for their resistance to 10 antibiotics by agar disk diffusion method. The most predominant specie as identified was E. faecium (43.1%), followed by E. *faecal is(223%),* while 34.6% were grouped as *Enterococcns* spp. High frequencies of resistance were observed among the isolates for erythromycin (80%), quinupristine/ dalfopristine (72.3%) and ciprofloxacin (72.3%) with vancomycin having the lowest resistance rates (43.1%). A total of 121 isolates were multiple resistant, with 11 being resistant to all 10 antibiotics tested. The multiple antimicrobial resistances (MAR) index therefore ranged from 0.3 to 1.00. The isolates exhibited a high level of variability with 85 resistant patterns identified among them. These isolates could serve as active reservoirs for antimicrobial resistance and resistance genes. Improved hygiene practices and controlled use of antibiotics in agriculture and animal husbandry are therefore desirable for environmental management and public health protection.

Keywords: enterococci, antimicrobial agents, poultry, antimicrobial resistance, vancomycin

1. Introduction

Antimicrobials are utilized in agriculture for veterinary medicine, as feed additives, and as biocides in crop and fruit production. They may also be used as growth-promoting and prophylactic agents in animals. Bacitracin, chlortetracycline, tylosin, avoparcin, neomycin, oxytetracycline and others are used for this purpose (Lukasova and Sustackova, 2003). Inappropriate use of these growth-promoting antibiotics is the major contributor to the emergence of antibiotic-resistant bacteria. In the European Union and many other countries, drugs that have been registered for therapeutic use in humans and/oranimals are not allowed to be used as growth-promoters.

However, many of the compounds used for growth-promotion are analogues of and show cross resistance with therapeutic antibiotics (Lukasova and Sustackova, 2003). The major agricultural use of antimicrobials is in the production of poultry, swine, and cattle, but antimicrobials are also used in aquaculture and there are limited uses for it in plants (Silbergeld*et al.*, 2008).

Chickens are extensively reared in close proximity to human habitation in Nigeria and can thus play an important role in the contamination of the environment with pathogens as well as serving as an important vehicle for the transfer of these pathogens to humans through the handling and consumption of its meat (Otalu*et al.*,

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2011). Poultry production has an intensive manufacturing cycle and thus one can hardly dispense with antimicrobial agents. This leads to increased antimicrobial resistance of pathogenic and commensal microbiota (Ruzauskas*et al.*, 2009). Antibiotics are extensively used as growth promoters in poultry production or to control infectious disease. Antimicrobial use and/ or especially abuse are considered to be the most vital selecting force to antimicrobial resistance of bacteria (Akond*et al.*, 2009; Lertworapreecha*et al.*, 2011). Avoparcin has been fed to broiler chickens, swine, and cattle. It causes cross-resistance to vancomycin and teicoplanin among bacteria (Sood*et al.*, 2008).Vancomycin and teicoplanin are used for the treatment of infections caused by Gram positive bacteria (enterococci) in case of resistance or allergy to β-lactams. These antibiotics act by blocking cell wall formation and resistance is due to synthesis of modified late peptidoglycan precursors (Lukasova and Sustackova, 2003).

Poultry are increasingly being associated with carriage of multi-resistant organisms that may cause disease in humans.Among these organisms are the enterococci (Lukasova and Sustackova, 2003). The enterococci have emerged as important opportunistic pathogens with a remarkable capacity of expressing resistance to several groups of antimicrobial agents, limiting the number of therapeutic options. They are associated with a variety of human infections, acquired mainly in the nosocomial setting, such as bacteremia, endocarditis, urinary tract infections and wound infections (Chapin *et al.*, 2005; Fracalanzza*et al.*, 2007; Sood*et al.*, 2008). On the other hand, species of the genus *Enterococcus* comprise a large proportion of the normal microbiota associated with the gastrointestinal tract of humans and animals (Fracalanzza*et al.*, 2007).

The use of antimicrobials for growth promotion in poultry production environments may therefore facilitate the dissemination of resistance to *Enterococcus* spp. that have the potential to be clinically significant (Lukasova and Sustackova, 2003). Most of the antibiotics used for agricultural purposes are only partially metabolized by animals and are then discharged through fecal contents either into sewage disposals or directly into rivers near animal farms. Several kinds of bacterial species displaying multiple antibiotic resistances (MAR) patterns, mainly *Escherichia coli* and enterococci, have been well documented in aquatic environments contaminated with animal fecal contents (Rho *et al.*, 2011). Consequently, the antibiotics used in agriculture are responsible for the increase in the prevalence of MAR in animal farming aquatic environments and may be directly linked to the antibiotic resistance problems in humans either via direct contact or through the food chain (Rho *et al.*, 2011).

Presently, there are no published data on multiple resistances in enterococciisolates from poultry samples in Nigeria. This report therefore serves as a baseline study on the prevalence and multidrug resistance in enterococci isolated from poultry farms in Imo State, Nigeria

2.0 Materials and Method

2.1. Collection of samples

A total of seventy five (75) samples were collected from four different poultry farms in Owerri, Nigeria. Samples included cloacal swabs, fecal and litter. Cloacal samples were collected from different birds per each poultry house using sterile swab sticks. Litter and fecal samples were collected from each poultry house using sterile plastic bags. The samples were collected by aseptic techniques to avoid sample contamination and were sent to the laboratory and analyzed within 2 hours of collection.

2.2. Isolation and Identification of Enterococci

The spread plate technique was adopted for the isolation of enterococci from the samples. A 10 fold serial dilution (one in ten dilutions) of each fecal and litter sample

was obtained to have discrete colonies of microorganisms on agar plates. An aliquot of 0.1-0.2ml of the sample was transferred using a sterile pipette onto the surface of sterile petri dishes containing the media. For the cloacal swabs, 1ml of sterile distilled water was poured into the cloacal swab containers, shaken and about 0.1ml inoculated onto the surface of the sterile petri dishes containing the media, and spread with a sterile hockey stick. The petri dishes were placed in the incubator at 44^{0}C for 24-48hours. The Slanetz and Bartley agar (Oxoid, CM UK) selective for the culturing of *Enterococcus* was used for culturing. Enterococci appeared as typical red or pink colonies on the medium. After incubation, 2-3 individual colonies from each petri dish were randomly selected and purified to obtain pure colonies by sub-culturing onto different Slanetz and Bartley agar plates and incubated at 37^{0}C for 24hours. The pure culture isolates were stored on agar slants and used for the different biochemical, morphological and antibiotic susceptibility tests. Isolates were primarily grouped based on their morphology, motility, catalase production, hemolysin production, pigmentation and ability to grow at 10°C, 45°C in 6.5% NaCl. The second step was identification of enterococci species following the recommendations of Manero and Blanch, (1999) by using their fermentation properties on sucrose, mannitol, arabinose, lactose, glucose, maltose, and sorbitol. Carbohydrate tests produced a yellow color for a positive result and a purple color for a negative result.

2.3. Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted on isolates using antibiotics selected on the basis of their importance to human medicine and use in poultry production. All isolates were tested for susceptibility to different antimicrobials using the Kirby-Bauer disk diffusion method as described by the National Committee for Clinical Laboratory Standard Guidelines (CLSI, 2007) and Bauer *et al.*, (1966). Each of the Enterococciisolate was inoculated into nutrient broth overnight at 37°C before testing. The turbidity of the actively growing culture was adjusted to correspond with that of a Barium sulphate (0.5 McFarland standards) standard. Subsequently 0.1 ml of the nutrient broth culture was inoculated onto Mueller Hinton agar plates (90mm diameter disposable petri dishes) and spread over the surface with sterile cotton swabs. The antibiogram consisting of 10 antibiotics (Oxoid, CM UK) encompassing seven antibiotic classes: penicillin (oxacillin-OXA, penicillin G-PEN), glycopeptides (vancomycin-VAN), fluoroquinolone (ciprofloxacin-CIP), macrolide erythromycin-ERY), streptogramin (quinipristine-dalfopristine-Q/D), aminoglycoside (streptomycin-STR, kanamycin-KAN, gentamicin-GEN) and tetracycline (tetracycline-TET) were then placed on the surface of each plate by means of antibiotic disk dispenser. Five discs were placed in each petri dish. Within 15 minutes of the application of the discs, the plates were inverted and incubated at 35°C. After 18 hours of incubation, inhibition zone diameters were measured using a transparent ruler and correlated into sensitive (S), or resistant (R) based on the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2007). The frequency of antibiotic resistance (%) in a population was calculated as the number of isolates showing resistance divided by the total number of enterococci isolates tested multiplied by 100.

2.4. Determination of Multiple Antibiotic Resistance Index (MAR)

Multiple antibiotic resistance index (MAR) was determined using the formula MAR=x/y, where x is the number of antibiotics to which test isolate displayed resistance and y is the total number of antibiotics to which the test organisms were exposed to (Mallo*et al.*, 2014).

2.5. Statistical Analysis of Results

Frequencies and percentages were calculated for study variables. Chi-square ($\chi 2$) test was used to calculate probabilities and determine significance. A p-value of less than or equal to 0.005 and 0.05 was considered to be statistically significant ($p \leq 0.005$) and ($p \leq 0.05$), while p-value more than 0.005 and 0.05 was considered to be statistically not significant (NS).

Results and Discussion

A total of one hundred and thirty enterococcal isolates were obtained from the seventy five (75) samples examined. Of the 130 isolates obtained, the highest frequency of 45(34.62%) was obtainedfrom the chicken cloacal swabs while litter from turkey produced the least number of 4 (3.08%) isolates (table 1).

The morphological characteristics of the enterococci colonies isolated were observed based on their pattern of growth, color, odor after incubation on the appropriate media. This shows the colonial appearance of the isolates on the media. Enterococci appeared as typical red or pink colonies on Slanetz and Bartley agar. The results obtained from the biochemical tests are shown in table 2. The microbiologically and biochemically positive strains not identified by sugar fermentation test were designated as *Enterococcus* spp.

Eighty-five (85) isolates were differentiated and identified as either *E. faecium* or *E. faecalis*. The most common species identified was *E. faecium*56 (43.1%) and *E. faecalis* 29(22.3%). It shows that *E. faecium* occurred more frequently than *E. faecalis* (Table 3).*E. faecium*was the predominant species recovered from the poultry samples, and is similar to findings of Knudtson and Hartman (1993) and Franz *et al.* (2003).

Antimicrobial resistant enterococci of both species and also the undifferentiated species were isolated from poultry litter, feces and cloacal swabs in this study. High frequencies of resistance were observed for erythromycin (80%), quinupristine /dalfopristine (72.3%) and ciprofloxacin (72.3%) (Table 4). Hayes *et al.*, (2004) conducted a large study on antibiotic resistance and its development within the broiler poultry house, and his results showed that multidrug resistance was observed in 53% of *Enterococcus faecium*and *Enterococcus faecalis*isolated from poultry litter and transport cages. These results suggest that animals and its wastes are possible reservoirs of multi-antibiotic resistant enterococci.

Resistance against MLS-antibiotics (macrolide, lincosamide and streptogramin) like erythromycin and quinupristin–dalfopristin (Synercid) is quite common in enterococci from animals fed with related antibiotics as APE (antimicrobial performance enhancer) like tylosin (a macrolide) or virginiamycin (a combination of two pristinamycins like quinupristin–dalfopristin) (van de Boggard*et al.*, 2000). The high percentage of erythromycin-resistant enterococci in the poultry could be as a result of tylosin present in the feed as growth promoting and prophylactic agents. Hammerum*et al.*, (1998; 2004; 2009) and Donabedian*et al.*, (2006) isolated vancomycin-resistant enterococci, quinupristin/ dalfopristin-resistant *E. faecium*, and gentamicin resistant enterococci from food of animal origin and from fecal samples from food animals. The findings of resistance to vancomycin and quinupristin/dalfopristin in enterococci of animal origin have been ascribed to the use of these antimicrobial agents as growth promoters in animal feed.

A relatively high frequency of resistance was observed for oxacilllin (69.2%), tetracycline (67.7%), streptomycin (67.7%), kanamycin (66.9%) and gentamicin (61.5%). For other compounds, the frequency was less than 61.5% with vancomycin having the lowest resistance rate (43.1%). *E. faecium*had the highest frequency of antimicrobial resistance among the antibiotics tested except in vancomycin and erythromycin (Table 4).

There was a high prevalence of tetracycline resistant phenotypes among the poultry isolates. Enterococci however have

been found to be naturally resistant to aminoglycosides, tetracyclines and erythromycin. The result in this study revealed a widespread resistance to macrolides, kanamycin, streptomycin and tetracycline. This has been found among isolates of *E. faecalis*and *E. faecium* isolated from humans, broilers and pigs as reported by Aarestrup*et al.*, (2000).Furtula*et al.* (2013) also reported a high level of resistance to tetracycline in enterococci isolates from poultry litter. Tetracycline has also been used widely in therapy and to promote feed efficiency in animal production systems since its approval in 1948. Tetracycline is one of the most commonly used antibiotics in veterinary medicine for the treatment of infections or as growth promoters on animal farms (Chopra and Roberts, 2001; Connell *et al.*, 2003; Rho *et al.*, 2011).

Vancomycin resistance was also high among the isolates. Vancomycin resistance is a significant point of concern when discussing antimicrobial resistance in *Enterococci* (Sood*et al.*, 2008; Butaye*et al.*, 2003). Itis especially widespread among isolates from farm animals, which may serve as a reservoir for vancomycin-resistant enterococci. These bacteria may enter the human food chain and are one potential source of superbug emergence (Rho *et al.*, 2011). Avoparcin, an antibiotic used only for growth promotion in animals, causes cross-resistance to vancomycin and teicoplanin among bacteria (Sood*et al.*, 2008; Butaye*et al.*, 2003). The most common vancomycin-resistant species was *E. faecalis;* similar to the report by Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR) (1999).

All antibiotics are freely available for use with animals without veterinary prescription. There is no monitoring by any regulatory authority of the use of antibiotics in agriculture. Thus, the addition of antimicrobial agents to animal feed or water for therapeutic or preventive purpose is uncontrolled (You *et al.*, 2006). The abusive use of antibiotics in the farms may be the cause of the high prevalence of antibiotic resistance that was found among the Enterococci isolates from poultry. At least 17 classes of antimicrobial agents are approved for growth promotion and feed efficiency, including tetracyclines, penicillins, macrolides, lincomycin (analog of clindamycin), and virginiamycin (analog of quinupristin/dalfopristin) in food animals (Florini*et al.*, 2005; Price *et al.*, 2007). Six of the eight classes of antibiotics screened in this study (penicillin, glycopeptides, tetracyclines, macrolides, aminoglycosides, and streptogramins) are used in poultry production; all of these drugs are categorized by the U.S. Food and Drug Administration as critically or highly important to human medicine (USFDA, 2003).

The most predominant resistant pattern was ErVaPnTeCiQdOxGeStKn and this was exhibited by eleven (11) isolates. The results also showed high level of variability in resistance amongst the resistant enterococci isolates, making it more difficult to choose a course of antibiotic therapy in the event of infection with these isolates.

Multidrug antibiotic resistance (MAR) index of the antimicrobial resistant enterococci ranged from 0.3 to 1.00. This index showed that the isolates were exposed to several antimicrobial drugs. It also revealed that there was widespread resistance in the environment. MAR index to 10 antibiotics revealed that 121 isolates were resistant to at least three drugs of the following antibiotics: erythromycin, ciprofloxacin, quinupristin/dalfopristin, penicillin, vancomycin, streptomycin, kanamycin, gentamicin, oxacillin and tetracycline, all of which are approved for use in animal production for growth promotion. Twenty-six (26) isolates had the highest frequency of 21.5% and were resistant to nine antimicrobial drugs. The lowest frequency observed was 7 (5.79%) and they belonged to the 4 antimicrobial drugs (table 6). Multidrug resistant enterococci isolated (121 isolates) showed an increasing antimicrobial resistance.

Due to the number of isolates resistant to common antibiotics as identified in the study, it is necessary to reevaluate the use of therapeutic antibiotics in stock farms (especially poultry farms) especially at the national and then regional levels. This data would serve as an indirect guide toassist in the control of antibiotic use in animal husbandry. In combination with proper hygiene and manufacturing aspects, a careful study and use of antibiotics in farm animals may prevent further proliferation of antibiotic resistant bacteria and possibly their determinants in our foods.

Table 1: Prevalence of enterococci isolatesfrom poultry

Sources of samples	No (%) of samples (n=75)	No (%) of isolates (n=130)
Chicken		
CloacalSwab	26 (34.67)	45 (34.62)
Litter	18 (24.00)	30 (23.08)
FecalMaterial	16 (21.33)	29 (22.31)
Turkey		
Cloacal Swab	5 (6.67)	11 (8.63)
Litter	4 (5.33)	4 (3.08)
FecalMaterial	6 (8.00)	11 (8.63)

Table 2: Biochemical tests used for identification of enterococci

Biochemical Test Properties	*Enterococcus faecium*	*Enterococcus faecalis*	*Enterococcus spp.*
Colony color	Reddish-pink	Reddish-brown with metallic sheen	Reddish-brown with metallic sheen
Gram stain	positive: cocci in pairs & chains	positive: cocci in pairs & chains	positive: cocci in pairs & chains
Catalase	-	-	-
Glucose	+	+	+
Sucrose	+	+	+
Mannitol	+	+	+/-
Maltose	+	+	+
Sorbitol	-	+	+/-
Arabinose	+	-	+/-
Growth at 10°C	+	+	+
Biochemical Test Properties	*Enterococcus faecium*	*Enterococcus faecalis*	*Enterococcus spp.*

Growth at 45°C	+	+	+
Alphahemolysis	+	+	+/-
Motility	-	-	+/-
Yellowpigment	-	-	+/-

Key: +:positive; - : negative

Table 3: Frequency (%) of different *Enterococcus* species isolated from poultry

Species	No (%) of Isolates (n=130)
E. faecium	56 (43.1)
E. faecalis	29 (22.3)
*Enterococcus*spp	45 (34.62)

Table 4: Frequency (%) of Resistant Enterococci Isolates from the Poultry Environment

	No (%) of Resistant Isolates			
Antibiotics	*E. faecium* (n=56)	*E. faecalis* (n=29)	*E. spp.* (n=45)	**Total** (n=130)
ERY	48 (85.7)	26 (89.7)	30 (66.7)	104 (80.0)*
Q/D	44 (78.6)	19 (65.5)	31(68.9)	94 (72.3)*
CIP	43 (76.8)	21(72.4)	30 (66.7)	94 (72.3)*
OXA	41(73.2)	19 (65.5)	30 (66.7)	90 (69.2)
TET	41 (73.2)	17 (58.6)	30 (66.7)	88 (67.7)
STREP	40 (71.4)	18 (62.1)	30 (66.7)	88 (67.7)
KAN	43 (76.8)	17 (58.6)	27(60.0)	87 (66.9)
GEN	34(60.7)	17 (58.6)	19 (64.4)	80 (61.5)
PEN	32 (57.1)	13(44.8)	25 (55.6)	70 (53.8)
VAN	23 (41.1)	14 (48.3)	19 (42.2)	56 (43.1)

Key: Gen- Gentamicin; Van- Vancomycin; Pen- Penicillin G; Kan- Kanamycin; Oxa- Oxacillin; CIP- Ciprofloxacin; Tet- Tetracycline; Q/D- Quinupristine/ Dalfopristine; Ery-Erythromycin; Strep- Streptomycin. **p≤0.005*

Table 5: Antimicrobial Resistance Patterns in Enterococcifrom Poultry

Antimicrobial Patterns	Frequency	Antimicrobial Patterns	Frequency	Antimicrobial Patterns	Frequency	Antimicrobial Patterns	Frequency
TeKn Ge1Ox Er Q/D 1		Pn Q/D Ox	1	Pen ErTe	1	ErTeCp	1
ErCp Ox	1	PnEr Ox	1	PnErGe	1	PnGe St Ox	1
PnErVn Ox	1	ErCp Q/D St	2	KnErTe Ox	1	Cp Q/D St Vn	1
ErTeGeVn	1	KnTe Q/D Ge St	1	ErTe Q/D St Ox	1	PnKnCp St Ox	1
Cp Q/D GeVn Ox	1	PnErCp Q/D Ox	1	KnErTeGe St	1	PnKn TE CpVn	1
ErTe Q/D St Vn	1	PnKn Q/D St Ox	1	KnErTeCp St	1	KnErGe St Vn	1
ErCp Q/D Ge St	1	ErTeCpGeVn	1	Er Q/D Ge St Vn	1	KnEr Q/D Ge St	1
PnKnErCp St Ox	1	PnKnTeCpGe St	1	PnErTeCp St Ox	1	PnErTeCpVn Ox	1
PnKnCp Q/D Ge Ox 1		PnKnEr Q/D Ge St	1	PnKnEr St Vn Ox	1	KnErTe Q/D St Ox	1
KnErTeCp Q/D Ox	2	KnCp Q/D GeVn Ox	1	KnErTe Q/D Ge Ox	1	ErTeCpGe St Ox	1
ErTeCpGe St Vn	1	ErTeCp St Vn Ox	1	ErTeCp Q/D Ge St	1	TeCp Q/D Ge St Vn	1
ErTeCp Q/D Vn Ox	1	ErCp Q/D Ge St Ox	1	ErTeCpGe St Vn	1	PnKnEr Q/D St Vn Ox	1
PnKnErCp Q/D St Ox	1	PnKnErCp Q/D GeOx	1	PnKnTeCpGe St Ox	1	PnKnErTe Q/D Ge Ox	1
PnKnEr Q/D GeVn Ox	1	PnKnErTeCp Q/D Ox	1	PnErTe Q/D Ge St Vn	1	PnErTeCp Q/D Ge Ox	1
PnErTe Q/D Ge St Ox	1	KnErTeCp Q/D Ge St	1	KnErTeCp Q/D St Ox	1	KnErTeCp Q/D St Ox	1
KnErTeCp Q/D Ge Ox	1	ErTeCp Q/D Ge St Ox	1	ErTeCp Q/D Ge St Ox	1	PnKnErTeCp Q/D St Ox	3
PnKnTeCp Q/D St Vn Ox	2	PnKnErTeCp Q/D Vn Ox	1	PnKnErTeCp Q/D Ge St	1	PnKnTeCp Q/D St Vn Ox	1
PnKnTeCp Q/D Ge St Ox	1	KnErTeCp Q/D Ge St Vn	2	KnErTeCp Q/D GeVn Ox	1	KnErTeCp Q/D Ge St Ox	3
KnErTeCp Q/D St Vn Ox	2	PnKnErTeCp Q/D Ge St Ox	8	PnKnErCp Q/D Ge St Vn Ox	6	PnKnErTeCp Q/D GeVn Ox	1
PnKnErTeCp Q/D Ge St Vn	1	PnKnErTe Q/D Ge St Vn Ox	1	PnKnTeCp Q/D GE St Vn Ox	2	PnKnErTeCp Q/D St Vn Ox	1
PnKnErTeCpGe St Vn Ox	1	PnErTeCp Q/D Ge St Vn Ox	1	KnErTeCp Q/D Ge St Vn Ox	4	PnKnErTeCp Q/D Ge St Vn Ox	11

Key: Pn-Penicillin G; Kn-Kanamycin; Er-Erythromycin; Te-Tetracycline; Cp-Ciprofloxacin; Ox-Oxacillin; Q/D-Quinupristine/Dalfopristine; Ge-Gentamicin; St- Streptomycin; Vn-Vancomycin.

Table 6: Multiple Antimicrobial Resistance Index of Enterococci isolated from Poultry

No of Antibiotic	Multiple Antibiotic Resistance-(MAR) Index	No (%) of multiple resistant Isolates (n=121)
3	0.3	8 (6.6)
4	0.4	7 (5.79)
5	0.5	15 (12.4)
6	0.6	21 (17.4)
7	0.7	16 (13.2)
8	0.8	17 (14.05)
9	0.9	26 (21.5)
10	1.0	11 (9.1)

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