DOI: 10.1002/vms3.84

Original Article

Serovars and antimicrobial resistance of non-typhoidal *Salmonella* isolated from non-diarrhoeic dogs in Grenada, West Indies

Victor A. Amadi*, Harry Hariharan*, Gitanjali Arya†, Vanessa Matthew-Belmar*, Roxanne Nicholas-Thomas*, Rhonda Pinckney*, Ravindra Sharma* and Roger Johnson†

**Pathobiology Department, School of Veterinary Medicine, St. George's University, St. George's, Grenada, West Indies and †Office International des Epizooties (OIE) Salmonella Reference Laboratory, Public Health Agency of Canada, National Microbiology at Guelph, Guelph, Ontario, Canada*

Abstract

Non-typhoidal salmonellosis remains an important public health problem worldwide. Dogs may harbour *Salmonella* in their intestines and can easily shed *Salmonella* in the environment with the possibility of transmission to humans. Thus, monitoring is essential to understand the role of dogs in zoonotic transmission. The objectives of this study were to determine the shedding of *Salmonella* by owned, apparently healthy dogs in Grenada, West Indies, to identify the serovars, and to examine their antimicrobial susceptibility profiles. Faecal samples collected during August to October, 2016 from 144 non-diarrhoeic owned dogs were examined by enrichment and selective culture for the presence of *Salmonella* spp. Eight (5.6%) of the tested animals were culture positive, yielding 35 *Salmonella* isolates that belonged to six serovars of *Salmonella enterica* subspecies *enterica.* These were serovars Arechavaleta from two dogs, Arechavaleta and Montevideo from one dog, and Javiana, Rubislaw, Braenderup and Kiambu from one dog each. All these serovars have been reported as causes of human salmonellosis globally. Antimicrobial susceptibility tests on 35 isolates showed absence of resistance to the currently used drugs for cases of human salmonellosis, including ciprofloxacin and cefotaxime. One isolate (2.9%) was resistant to neomycin, two isolates (5.7%) showed intermediate susceptibility to neomycin, and another (2.9%) had intermediate susceptibility to tetracycline. This is the first report of isolation and antimicrobial susceptibilities of non-typhoidal *Salmonella* serovars from dogs in Grenada. This study shows that dogs in Grenada may be involved in the epidemiology of salmonellosis.

Keywords: Salmonella, dogs, serovars, grenada, antimicrobial susceptibility.

Correspondence: Harry Hariharan, Department of Pathobiology, School of Veterinary Medicine, St. George's University, St. George's, Grenada, West Indies. E-mail: hhariharan@sgu.edu

Introduction

Non-typhoidal salmonellosis is an important zoonosis worldwide. Due to considerable geographical and temporal variation in the prevalence and serovars of *Salmonella* spp. in animals and humans, monitoring is important to understand the role of animals in zoonotic transmission (Leonard 2014). It has been well known for several decades that dogs may carry *Salmonella* spp. in their intestinal tracts, and an asymptomatic carrier state and less commonly clinical salmonellosis can occur, with the possibility of transmission to humans (Wolf *et al.* 1948; Mackel *et al.* 1952; Morse & Duncan 1975; Morse *et al.* 1976). Generally, faecal shedding has been less frequent in household pet dogs, compared to those in kennels and stray dogs (Carter & Quinn 2000). Studies of apparently healthy dogs conducted decades ago identified many different serovars and carrier rates from 4 to 16% (Morse *et al.* 1976; Shimi *et al.* 1976). More recent information on the carrier state in dogs is available from the United States (Jay-Russell *et al.* 2014; Leahy *et al.* 2016; Reimschuessel *et al.* 2017), Ethiopia (Kiflu *et al.* 2017), the United

Kingdom (Philbey *et al.* 2014), Thailand (Srisanga *et al.* 2016), Taiwan (Tsai *et al.* 2007), Turkey (Kocabiyik *et al.* 2006; Bagcigil *et al.* 2007), Trinidad (Seepersadsingh *et al.* 2004) and Germany (Weber *et al.* 1995). This study was conducted to determine the prevalence and serovars of non-typhoidal *Salmonella* spp. in non-diarrhoeic dogs in Grenada, and the susceptibility of isolates to 12 antimicrobials including those used frequently to treat salmonellosis in humans.

Materials and Methods

This study had the approval of the St. George's University Institutional Animal Care and Use Committee (IACUC 15006-R). Prior to entering dogs into the study, the dog owners were asked to review and sign a consent form which describes the study and its purpose. Dog owners were randomly selected from the six parishes of the island of Grenada based on their availability and the acceptance of the owners to include their dogs in this study. The distribution of the tested dogs are: St. George's parish (n = 32), St. David's parish (n = 28), St. Andrew's parish (n = 26), St. Patrick's parish (n = 24), St. Mark's parish (n = 16) and St. John's parish (n = 18). For each participating dog, the gender, age, housing (indoor/outdoor or strictly indoor), breed, health history, history of antibiotic use and date of sampling were recorded. In terms of the housing of the dogs, the indoor/outdoor dogs referred to those dogs that were kept in cages but were allowed to roam around, while the strictly indoor dogs were those kept in homes and not allowed to roam. Participating dogs were sampled from August to October, 2016. A fresh faecal sample was collected from each dog by inserting a gloved finger into the rectum (Bassert & Thomas 2014). Approx. 1–2 g of each sample was placed in a vial of transport medium (Cary Blair Transport Medium with Indicator, 15 mL/Vial, Remel, Lenexa, KS 66215). The vial was agitated to mix the sample with the transport medium, placed in a cooler with ice packs and transported to the Bacteriology Laboratory, School of Veterinary Medicine, St. George's University for laboratory analysis. The time between sample collection and culture was approximately 2 h.

For the isolation of *Salmonella*, established culture methods (Gorski *et al.* 2011) were used with slight modifications described by Drake *et al.* (2013) and Sylvester *et al.* (2014a). The contents of each vial were mixed thoroughly and an aliquot (1 mL) was transferred into a tube containing 10 mL of tryptic soy broth (TSB) (Remel, Lenexa, KS) and incubated at 37°C for 18–24 h. After incubation, 100 μL of the TSB culture was inoculated into Rappaport-Vassiliadis broth (RVB) (Difco/BD, Sparks, MD) and incubated at 42°C for 48 h. An aliquot of the RVB culture was then sub-cultured on xylose lysine deoxycholate agar (XLD) (Difco/BD) plates and incubated at 37°C for 18–24 h. To increase the chances of isolation of multiple serovars, up to five individual colonies with typical *Salmonella* morphology on XLD agar plates (red colonies with a black centre) were selected and sub-cultured a second time on XLD agar plates and incubated at 37°C for 18–24 h to obtain pure colonies. After incubation, single colonies from the second XLD plates were streaked onto tryptic soy agar (TSA) (Difco/BD) and incubated at 37°C for 18–24 h. The resulting colonies from each TSA plate were tested for agglutination with *Salmonella* O antiserum poly A-I & Vi (Difco/BD). All the agglutination-positive isolates resembling *Salmonella* were inoculated into API-20E®(Analytical Profile Index, Bio-Merieux Inc. Durham,NC) strip and incubated at 37°C for 18–24 h for identification as *Salmonella* spp. Identified pure *Salmonella* cultures were stored in 10% sterile skim milk solution at −80°C until shipped by air on dry ice for serotyping. Reference strain of *S*. Typhimurium ATCC 14028 was used as a quality control. All isolates were serotyped at the World Organization for Animal Health (Office International des Epizooties; OIE) Salmonella Reference Laboratory of the Public Health Agency of Canada's National Microbiology Laboratory at Guelph Ontario, Canada, using established methods (Shipp & Rowe 1980; Ewing 1986). The serovars were named as per the antigenic formulae listed by Grimont (2007).

All the *Salmonella* isolates were tested for susceptibility to 12 antimicrobials: amoxicillin-clavulanic acid (20/10 μg), ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), neomycin (30 μg), tetracycline (30 μg) and trimethoprim-sulfamethoxazole (1.25/23.75 μg) using the standard Kirby–Bauer disc diffusion method on Mueller Hinton agar (Difco/BD) following recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2015). The inhibition zone sizes were interpreted based on CLSI guidelines. *Escherichia coli* ATCC 25922 was used as quality control strain (Eguale *et al.* 2015).

Statistical Methods

The differences in the proportions of female vs. male dogs, indoor/outdoor dogs vs. strictly indoor dogs, and <1 year vs. >1 year dogs that were culture positive for *Salmonella* spp were compared using chi-squared (χ^2) analysis created in an online data analysis software: The OpenEpi-Two by Two Table (http://www.openepi.com/Menu/OE_Menu.htm). The level of statistical significance was set at alpha equal to 0.05 ($\alpha = 0.05$). A value of $P < 0.05$ was considered statistically significant.

Results

One hundred and forty-four non-diarrhoeic owned dogs were enrolled in the study. They comprised 140 (97%) indoor/outdoor dogs and four (3%) strictly indoor dogs. By gender, 56 (39%) were female and 88 (61%) were male, and by age, 46 (32%) were less than 1 year (<1) and 98 (68%) were greater than 1 year (>1). All the tested dogs were mixed breed dogs known colloquially as pothounds. None of the dogs had a history of diarrhoea or vomiting 3 weeks prior to sampling. Ten (7%) of the dogs had been treated with antibiotics 2 weeks prior to sampling; eight with doxycycline, one with cephalexin and one with amoxicillin.

Salmonella spp were isolated from the faecal samples of eight of the 144 dogs (5.6%). These eight dogs included three (3.4%) of 88 male dogs and five (8.9%) of 56 female dogs, seven (5%) of 140 indoor/outdoor dogs and one (25%) of four strictly indoor dogs, four (9%) of 46 < 1 year dogs and four (4%) of 98 > 1 year dogs (Table 1). There were no significant differences between the proportions of *Salmonella*-positive female and male dogs ($P = 0.302$), indoor/outdoor and strictly indoor dogs ($P = 0.5386$) or <1 year and >1 year dogs ($P = 0.4612$). None of the dogs with history of antibiotic treatment were *Salmonella* positive.

Selection of up to five colonies with typical *Salmonella* morphology from each of the eight positive samples led to a total of 35 confirmed *Salmonella* isolates; two from one sample, four from two samples and five from five samples. On serotyping, these 35 *Salmonella* isolates belonged to six *Salmonella enterica* subsp *enterica* serovars: Arechavaleta ($n = 13$), Braenderup ($n = 5$), Javiana ($n = 2$), Kiambu ($n = 5$), Montevideo ($n = 5$) and Rubislaw ($n = 5$) (Table 1).

Of the eight *Salmonella*-positive dogs, three (37.5%) were positive for serovar Arechavaleta, one of which also carried serovar Montevideo. The remaining five dogs were each positive for one of serovars Braenderup, Javiana, Kiambu, Montevideo and Rubislaw, one dog each (Table 1).

Based on results of antimicrobial susceptibility testing by the Kirby-Bauer assay, 34 (97.1%) of the 35 *Salmonella* isolates were not resistant to the tested antimicrobials. One (*S*. Montevideo) (2.9%) was resistant to neomycin. Two isolates (*S*. Montevideo and *S*. Javiana) (5.7%) showed intermediate susceptibility to neomycin and another (*S*. Montevideo) (2.9%) had intermediate susceptibility to tetracycline.

Discussion

In the present study, *Salmonella* was isolated from 8/144 dogs (5.6%). There is a possibility that the culture-negative dogs in the present study may still be sub-clinical shedders of *Salmonella*, because faecal shedding can be intermittent (Carter & Quinn 2000). Also, some *Salmonella* isolates may have been missed because of using only one selective medium,

Table 1. *Salmonella* serovars isolated from eight non-diarrhoeic owned dogs in Grenada.

Dog ID	Age	Gender	Housing	No. of *Salmonella* isolates	Serovars* (n)
D13	>1 year	Male	Indoor/outdoor	5	*S*. Kiambu (5)
D30	<1 year	Male	Strictly indoor	5	*S*. Arechavaleta (5)
D33	<1 year	Female	Indoor/outdoor	4	*S*. Arechavaleta (3) *S*. Montevideo (1)
D36	>1 year	Female	Indoor/outdoor	2	*S*. Javiana (2)
D51	<1 year	Male	Indoor/outdoor	4	*S*. Montevideo (4)
D96	>1 year	Female	Indoor/outdoor	5	*S*. Arechavaleta (5)
D130	<1 year	Female	Indoor/outdoor	5	*S*. Rubislaw (5)
D133	>1 year	Female	Indoor/outdoor	5	*S*. Braenderup (5)

*One dog (D33) was positive for two serovars: *S*. Arechavaleta and *S*. Montevideo.

and not including hydrogen sulphide-negative isolates which are increasing in prevalence (Lin *et al.* 2014; Leonard *et al.* 2015). In a study of non-diarrhoeic dogs across Trinidad, 3.6% were positive for *Salmonella* spp. (Seepersadsingh *et al.* 2004). Recently, in dogs sampled from animal shelters across Texas, USA, the prevalence of faecal shedding of *Salmonella* was 4.9% (Leahy *et al.* 2016). It is obvious that dogs can be reservoirs of many different serovars in both temperate and tropical areas (Carter & Quinn 2000).

It is pertinent to note that the island of Grenada is a small developing country where only few individuals own and care for their dogs as pets. Majority of dog owners keep their dogs for security and hunting purposes. The dogs live in close proximity to each other and to human homes. They are usually allowed to roam around, mingle with other dogs and scavenge for food and may travel from one parish to another. Thus, the possibility of intermingling between the dogs is high. Because Grenada dogs are allowed to roam, they may be readily exposed to pathogenic organisms in the environment which may have contributed to the high percentage of *Salmonella* detected in the indoor/outdoor dogs.

Unlike in developed countries where the majority of the dogs are pure breeds and fed with commercial pet diet, the majority of the dogs in Grenada are mixed breed dogs (pothounds) and mainly fed with cooked homemade food such as rice, chicken, beef, etc. Due to the disorganization and unsystematic distribution of the dogs in the small island of Grenada, the relationship between the *Salmonella*-positive dogs and their distributions was not determined. Also, because the tested dogs were fed with random homemade food and were capable of scavenging for food, the relationship between the *Salmonella*-positive dogs and their diet was not determined. Furthermore, all the tested dogs were mixed breed dogs, hence, the relationship between the *Salmonella*-positive dogs and their breed was not determined in this study.

In the present study, six serovars were isolated, with *S*. Arechavaleta predominating. The major *Salmonella* serovars associated with human disease (Enteritidis and Typhimurium) were not isolated. A more extended study of dogs in Grenada is required to understand the significance of this finding. In a recent multilaboratory survey in the United States (Reimschuessel *et al.* 2017), *S*. Newport was the most common isolate (21% of total) from dogs. *S*. Enteritidis consisted of 8%, and *S*. Typhimurium 6%. In a recent study on *Salmonella* from household dogs in Ethiopia, *S*. Bronx and *S*. Newport were the most common serovars, with rates of 17% and 14%, respectively. Other serovars were Typhimurium, Indiana, Kentuky, Saintpaul and Virchow, 9.5% each. In the long list of more than 40 serovars isolated from dogs in various countries; Germany, Iran, Ireland, South Africa, United Kingdom and United States from 1951 to 1988, (Carter & Quinn 2000) serovars Arechavaleta and Kiambu were not isolated from dogs in those countries. Serovar Arechavaleta was also not isolated from dogs in the recent

multilaboratory study in the United States. (Reimschuessel *et al.* 2017). *Salmonella* Arechavaleta has been isolated recently from a mongoose and a cane toad in Grenada (Drake *et al.* 2013; Miller *et al.* 2014). In a study of the prevalence of *Salmonella* in non-diarrhoeic dogs in Trinidad, 28 serotypes were isolated, with Arechavaleta comprising 10% of the isolates (Seepersadsingh *et al.* 2004). This serovar was responsible for an outbreak of human gastrointestinal illness in 48 people in Trinidad nearly four decades ago, apparently from contaminated water (Koplan *et al.* 1978). Although this serovar is not a common cause of human infection, it has caused 80 confirmed cases of salmonellosis from 1999 to 2009 in the United States (CDC 2009).

Serovar Montevideo comprised 42%, 36% and 25% of the *Salmonella* isolates from mongooses, blue land crabs and cane toads, respectively, in Grenada (Drake *et al.* 2013; Peterson *et al.* 2013; Sylvester *et al.* 2014a). Therefore, it is not surprising that one dog was positive for this serovar in the present study. Other studies in different geographical areas have shown dogs carrying this serovar. For instance, S. Montevideo was isolated from the rectal swabs of 50 foxhounds from a pack of 61 in UK. The infection apparently spread by scavenging of dead sheep and aborted foetuses. The organism also was isolated from the aborted foetuses of a bitch (Caldow & Graham 1998). Schotte *et al.* (2007) reported an outbreak in military kennel dogs from a commercial feed contaminated with S. Montevideo. Several dogs had mild diarrhoea, but some were sub-clinical shedders with positive faecal cultures. Serovar Montevideo consisted of 5% of the isolates from dogs in the recent multilaboratory study in the United States. (Reimschuessel *et al.* 2017). Systemic and extraintestinal forms of human infection due to *S*. Montevideo have been reported from different parts of the world (Asseva *et al.* 2012; Rai *et al.* 2014).

In Grenada, *S*. Rubislaw was the most common serovar in wildlife, making up 59% of all *Salmonella* isolates from wild green iguanas and 33% of isolates from cane toads (Drake *et al.* 2013; Sylvester *et al.* 2014a), and has also been isolated from mongooses (Miller *et al.* 2014). This serovar has been reported as one of the most frequently isolated and persistent water-borne *Salmonella* in Georgia, United States (Haley *et al.* 2009; Maurer *et al.* 2015).

S. Javiana, one of the serovars isolated in the present study, was the most frequent serovar isolated from dogs in Trinidad (Seepersadsingh *et al.* 2004), and one of the most frequent isolates from cane toads and mongooses in Grenada. Serovar Javiana consisted of 8% of all isolates from dogs in a multilaboratory study in the United States recently (Reimschuessel *et al.* 2017). It is also widespread in shelter dogs in Texas, United States (Leahy *et al.* 2016). This serovar has a low infectious dose and possesses several virulence genes and plasmids that can contribute to large salmonellosis outbreaks in humans (Elward *et al.* 2006; Mezal *et al.* 2013).

Serovar Braenderup is not commonly associated with dogs, but it was once isolated in Iran (Shimi *et al.* 1976), and recently from a dog in Texas, United States (Reimschuessel *et al.* 2017). Recently, Nakao *et al.* (2015) reported outbreaks of human disease from *S*. Braenderup associated with a mail-order poultry hatchery in the United States. Also, it has been one of the prominent serovars in human clinical cases in Columbia (Rodriguez *et al.* 2016).

We isolated serovar Kiambu from one dog. This serovar has not been isolated so far from animals or humans in Grenada. However, it has been reported as a cause of clinical salmonellosis with positive blood cultures in children at the Children's Hospital, London, Ontario, Canada (Cellucci *et al.* 2010), and has been implicated in human infection from feral pigeons. It also has been isolated from beef samples in Morocco and from faecal samples of kangaroos in Australia (Haag-Wackernagel & Moch 2004; Bouchrif *et al.* 2009; Potter *et al.* 2011). Isolation of a novel *Salmonella* serovar (*S*. Kiambu) from a dog indigenous to a developing country like Grenada is important to understand the possible role of domestic animals in the spread of novel pathogenic *Salmonella* serovar in the environment and zoonotic transmission. Continuous monitoring is important in order to determine the risk factor for the emergence of novel *Salmonella* serovar.

Presently, there is no published information on human isolates of *Salmonella* in Grenada. Thus, there is no substantial evidence that suggests that

dogs may be a source of *Salmonella* for humans. The prevalence and serovars of human *Salmonella* need to be investigated to understand the role of dogs in the epidemiology of *Salmonella* in Grenada.

The antimicrobial susceptibility testing showed that antimicrobial resistance is minimal among the *Salmonella* isolates from dogs in this study, and limited to neomycin. The serovar that showed resistance to neomycin was Montevideo. Another isolate of this serovar showed intermediate resistance to neomycin, and one to tetracycline. One isolate of serovar Javiana also showed intermediate resistance to neomycin. Information on the resistance of *S*. Montevideo of dog origin is limited. However, it may be worthwhile to note emerging resistance to aminoglycosides and tetracyclines. Of 15 *S*. Montevideo isolates from dogs in United Kingdom, tested against 14 drugs including aminoglycosides and tetracycline, none showed resistance to any drug except sulfamethoxazole and trimethoprim (Philbey *et al.* 2014). In contrast, resistance to neomycin among *Salmonella* isolates from household dogs in Trinidad was 42% (Seepersadsingh *et al.* 2004). A recent study on antimicrobial drug susceptibility of *Salmonella* isolates from household dogs in Ethiopia showed resistance rates varying from 26% to 60% for amoxicillin-clavulanic acid, ampicillin, cephalothin, streptomycin, neomycin and oxytetracycline; with 50%, and 60% for the last two drugs (Kiflu *et al.* 2017). These findings seem to show that drug resistance of *Salmonella* from dogs can vary widely depending on the geographic area and possibly, serovars. Although one isolate of S. Montevideo in this study was found resistant to neomycin, previous studies on this serovar from blue land crabs and mongoose in Grenada showed no resistance. However, intermediate resistance to streptomycin was noted in one isolate from mongoose (Peterson *et al.* 2013; Miller *et al.* 2014). A study of *Salmonella* isolates from dogs in the UK showed no resistance to aminoglycosides, but 13% of Montevideo isolates were resistant to sulfamethoxazole (Philbey *et al.* 2014). Long-term studies in the future may elucidate drug resistance trends to aminoglycosides and other drugs.

The primary drugs of choice for non-typhoid salmonellosis in humans are ciprofloxacin and extended spectrum cephalosporins (Guerrant *et al.* 2001; Rabinowitz & Conti 2010), to which all our isolates were susceptible. Similar results were obtained with our studies on Salmonella isolates from cane toads and blue land crabs (Drake *et al.* 2013; Peterson *et al.* 2013). However, intermediate susceptibility to cefotaxime and tetracycline among isolates from green iguanas was of concern (Sylvester *et al.* 2014a). Combination therapy with cefotaxime and ciprofloxacin has been successful for non-typhoidal *Salmonella* bacteremia (Chang *et al.* 2006).

Resistance of enteric bacterial isolates to beta-lactams antibiotics is uncommon in Grenada. However, several studies have shown that tetracycline resistance is common among a variety of bacteria from different sources in Grenada (Sylvester *et al.* 2014b; Amadi *et al.* 2015a,b,c,d; Farmer *et al.* 2016). Tetracycline resistance is high in Grenada. It is noteworthy that chlortetracycline is routinely used as a feed additive for pigs in Grenada. Furthermore, oxytetracycline is used for treatment of pigs for bacterial infections (Sabarinath *et al.* 2011).

In conclusion, we estimated the prevalence of S*almonella* in the faeces of non-diarrhoeic owned dogs in Grenada to be 5.6%. The isolates were of six serovars with potential to cause human illness, four of which have been isolated recently from wildlife of this island nation. Antimicrobial resistance profiles indicated all isolates were susceptible to the antimicrobials of choice for the treatment of human salmonellosis. Dogs in Grenada may be a source of human exposure to *Salmonella*.

Acknowledgements

The authors gratefully acknowledge the assistance of Linda Cole, Ketna Mistry, Linda Nedd-Gbedemah, Ann Perets and Betty Wilkie of the OIE Salmonella Reference Laboratory, Public Health Agency of Canada, Guelph, Ontario for serotyping; and Dr. M. Lanza-Perea, Dr. K. Carter, Dr. K. Tiwari, Dr. L. Andrews, the clinicians and technicians at the St. George's University, Small Animal Clinic and Ms.

Jennifer Allen for their assistance with sample collection.

Source of funding

This work was funded by the St. George's University Small Research Grant Initiative (SRGI).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate review committee approval has been received. The St. George's University Institutional Animal Care and Use Committee's guidelines for Animal Care and Use were followed (IACUC 15006-R).

Contributions

Study design: VAA, RS, RP, HH. Sample collection: VAA. Sample testing: VAA, VM, RN. Serotyping: GA, RJ. Statistical analysis: VAA. Manuscript draft: VAA, HH, GA, VM, RN, RP, RS, RJ. Revision and manuscript approval: VAA, HH, GA, VM, RN, RP, RS, RJ.

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