Original Article

An Investigation into Enterobacteriaceae Responsible for Early Mortality in Japanese Quail Chicks and Their Antibiotic Susceptibility Patterns

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ABSTRACT

Quail is an alternative source of protein for humans. These birds can be affected by common bacterial infections. Bacterial contamination of egg is the most common cause of mortality in Japanese quail chicks. In order to study the role of some members of Enterobacteriaceae responsible for early mortality in Japanese quail chicks, 100 dead or moribund quail chicks were obtained from 10 different farms in Ahvaz, Iran. Samples were taken from the liver and yolk sac of the birds and bacterial isolation from samples was conducted by streaking them on MacConkey, Brilliant Green, Salmonella-Shigella and Xylose Lysine Deoxycholate agar plates. The plates were incubated at 37 °C for 24-48 hours, and by standard biochemical tests bacterial isolates were identified. Final confirmation of Salmonella serotypes was performed by Razi Institute. All the isolates were examined for susceptibility to 12 different antibiotics (Padtan-Teb Co., Tehran, Iran) by the disk diffusion (Kirby Bauer) method. The results showed that 78% of the quail chicks were infected. The isolated bacteria were Escherichia coli (44%), Klebsiella pneumonia (8%), Salmonella serovar ruzizi (5%), Salmonella serovar typhimurium (3%), Enterobacter cloacae (4%), Enterobacter aerogenes (4%), Proteus vulgaris (5%) and Proteus mirabilis (5%). One hundred percent susceptibility was observed to gentamycin, soltrim, tetracycline, fosfomycin, florfenicol, cephalexin and ceftriaxone. E. coli isolates were susceptible to soltrim and ceftriaxone, Salmonella isolates were susceptible to fosfomycin, Enterobacter isolates were susceptible to ceftriaxone and Proteus and Klebsiella isolates showed susceptibility to ceftriaxone. It is concluded that the members of Enterobacteriaceae family, specifically the genera Escherichia and Salmonella, are the major causes of early mortality in newly-hatched Japanese quail chicks.

Keywords: Japanese Quail, Mortality, Enterobacteriaceae, Antibiotic Susceptibility

Enquête sur les Entérobactéries Responsables de la Mortalité Précoce des Poussins de Caille Japonais et de leurs Profils de Sensibilité aux Antibiotiques

Résumé: La caille est une source alternative de protéines pour l'homme. Ces oiseaux peuvent être touchés par des infections bactériennes courantes. La contamination bactérienne des œufs est la cause la plus fréquente de mortalité chez les poussins de caille japonais. Afin d'étudier le rôle de certains membres des Entérobactéries responsables de la mortalité précoce chez les poussins de caille japonais, 100 poussins de caille morts ou moribonds ont été obtenus à partir de 10 fermes différentes de la ville d'Ahvaz (Iran). Des prélèvements ont été effectués aux niveaux du foie et de la vésicule vitelline des oiseaux et l'isolement bactérien a été réalisé en les striant sur des plaques de gélose MacConkey, Brilliant Green, Salmonella-Shigella et Xylose Lysine

Deoxycholate. Les plaques ont été incubées à 37 °C pendant 24 à 48 heures et des tests biochimiques standards ont permis d'identifier les isolats bactériens obtenus. La confirmation finale des sérotypes des salmonelles a été effectuée par l'Institut Razi. La sensibilité de chacun des isolâtes vis-à-vis de 12 antibiotiques différents (padtan-Teb Co., Téhéran, Iran) a été évaluée par la méthode de diffusion sur disque (Kirby Bauer). Nos résultats ont montré que 78% des poussins de caille étaient infectés. Les bactéries isolées étaient *Escherichia coli* (44%), *Klebsiella pneumoniae* (8%), *Salmonella sérovar ruzizi* (5%), Salmonella sérovar typhimurium (3%), *Enterobacter cloacae* (4%), *Enterobacter aerogenes* (4%), *Proteus vulgaris* (5%) et *Proteus mirabilis* (5%). On a observé une sensibilité de 100% des isolates vis-à-vis de la gentiamycine, du soltrim, de la tétracycline, de la fosfomycine, du florfénicol, de la céphalexine et de la ceftriaxone. Les isolats d'*E. coli* étaient sensibles à la soltrim et à la ceftriaxone alors que, les isolats d'Enterobacter de Proteus et Klebsiella. On peut en conclure que les membres de la famille des Entérobactéries, en particulier les genres Escherichia et Salmonella, sont les principales causes de mortalité précoce chez les poussins de caille japonais récemment éclos.

Mots-clés: Caille Japonaise, Mortalité, Entérobactéries, Susceptibilité aux Antibiotiques

INTRODUCTION

Quails, due to high growth rates and short generation interval, in addition to the production of meat and eggs, are also used as laboratory animals. Bacterial contamination in quail chicks is one of the limiting factors in breeding these birds. Quail meat and eggs have high quality and biological protein value with low calories (Chindo and Olowoniyan, 2006). For suitable control and preventive programs in quail industry, appropriate knowledge about the diseases affecting these birds is necessary. Poor breeder farm management and hatchery hygiene, as well as use of floor eggs are some of the predisposing factors to early mortality in quail chicks. Transmission of infection through the bloodstream and contamination of yolk sac have been reported as the routes of infection. The most common infectious diseases that occur in quail production are collibacillosis, salmonellosis, ulcerative enteritis, fowl cholera, quail bronchitis, Newcastle disease and quail pox (Dozier et al., 2010). Various microorganisms causing the infections are transmitted through the yolk sac. Salmonella and Escherichia coli (E. coli) are the most important factors in yolk sac infection. E. coli bacteria can penetrate through the oviduct and are common in egg shell contamination. In addition to Salmonella and

E. coli, other bacteria such as Proteus and Bacillus can also cause infection of the yolk (Khan et al., 2004). Pets, poultry and pigs are specifically considered the main hosts for Salmonella (Vo et al., 2006). In recent years, the incidence and severity of collocabilities has rapidly increased, and it is likely that this trend will continue and create even more problems in the poultry industry (Altekruse et al., 2002). Resistance to antibiotics is a major problem in humans and animals. The extensive use of these drugs has led to the selection of bacteria that are naturally resistant. Not only might these bacteria become major species population, but they may also genetically transfer this resistance to susceptible bacteria (Glynn et al., 1998). In this study we aimed to determine the antimicrobial drug susceptibility of Enterobacteriaceae responsible for the death of quail chicks in farms around Ahvaz, Iran.

MATERIAL AND METHODS

Bacteriology. One hundred dead or moribund quail chicks under 10 days of age were collected from 10 farms in Ahvaz. The history and clinical symptoms of these quail chicks were recorded. The samples were transferred to a laboratory at the earliest possible time. In the laboratory, dying chicks were killed by neck dislocation. The carcasses were dipped in a saline

solution and an autopsy was carried out under the hood and adjacent to the flame. Samples were taken from liver and yolk sac and streaked onto MacConkey (Merck, Germany), Brilliant Green (Himedia, India), Salmonella-Shigella (Himedia, India) and Xylose Lysine Deoxycholate (Himedia, India) agar plates and incubated at 37 °C for 24-48 hours. The representative colonies were sub-cultured onto blood agar (Merck, Germany) to verify their purity, and then they were staining identified using gram and standard biochemical tests, namely Triple Sugar Iron Agar (TSI) reaction, indole, H2S production, motility, methyl red, Voges-Proskauer, urease, phenylalanine deaminase agar (PD), lysine--decarboxylase sulfhydrase (LDS) and citrate utilization tests (Markey et al., 2013). The confirmation of Salmonella serotypes was performed by Razi Vaccine and Serum Research Institute.

Antibacterial Drug susceptibility Test. То determine antibiotic sensitivity of the isolated bacteria, disk diffusion test was conducted according to the existing guidelines. The following antibiotic discs (Pad Tan Teb Ltd., Iran (were used: cephalexin (CN 30µg), ciprofloxacin (CP5 µg), florphenicol (FF 30 µg), gentamycin (GM 120 µg), lincospectin (LST 200 µg), soltrim (SLT 23.75 µg), phosphomycin (FO 200 µg), cefotaxime (CTX 30 µg), ceftriaxone (CRO 30 µg), enrofloxacin (NFX 5 µg), doxycycline (D 30 µg) and oxytetracycline (T 30 µg). The isolates were cultured on nutrient agar (Merck, Germany) and incubated at 37 °C for 24 hours, then a single colony from the nutrient agar plates was transferred into a test tube containing 5 ml tryptic soy broth (Merck, Germany) and incubated at 37 °C for 2 to 8 hours until the turbidity matched the 0.5 McFarland turbidity standard. Then, a swab was inserted into the bacterial suspension and cultured on Muller-Hinton agar (Merck, Germany). The culture was allowed to adsorb for 10 minutes and then the antibiotic discs were placed on the plate at an appropriate distance from each other (24 mm). The plates were incubated at 37 °C for 18 hours. The inhibition zone diameters around the antibiotic discs were measured. The results were in accord with the standard inhibition zone diameters of individual antibiotic discs. Based on size of inhibition zones of various antibiotics, the isolates were classified as sensitive, intermediately sensitive or resistant according to the guidelines of the manufacturer of the antibiotic disc.

RESULTS

Bacteriological results. The results showed 78% contamination with Enterobacteriaceae. The isolated bacteria were *E. coli*, Salmonella serovar (ser.) Ruzize, Salmonella ser.typhimurium, *Proteus vulgaris*, Proteus mirabilis, *Klebsiella pneumonia*, *Enterobacter cloacae*, and *Enterobacter aerogenes* (Table 1). Forty-four of the 100 considered quail chicks were positive for *E. coli* infection. Totally, 47 *E. coli* isolates were collected (15 isolates from the liver and 32 isolates from the yolk sac). *E. coli* isolates were separated from all the samples that were taken from 10 different farms.

Table1. Bacteria isolated from liver and yolk sac of

100 dead quail chicks								
Bacteria	Yolk sac	Liver	Total					
E. coli	32	15	44					
Proteus	8	4	10					
Salmonella	9	2	8					
Enterobacter	7	2	8					
Klebsiella	7	3	8					

The most important biochemical properties of this bacterium include acid-acid reaction with gas production and without H2S production in TSI medium, green gloss in the Eosin Methylene Blue medium (Figure 1), urease negative, and with the IMViC formula positive, positive, negative, negative. Eight of the 100 quail chicks were positive for Salmonella, 11 isolates of Salmonella were separated. The most important biochemical characteristics of Salmonella consist of alkali-acid reaction with H2S production in TSI medium, LD positive, PD and urease negative, and with the IMViC formula negative, positive, negative, positive (Figure 2). The results of Razi Institute Salmonella serotyping (with letter number: 7072/250) showed that 8 of 11 isolates were categorized in ser. ruzizi (2 isolates from the liver and 6 isolates from the yolk sac) and three isolates of Salmonella were in ser.



Figure 1. E. coli green gloss in the Eosin Methylene Blue medium



Figure 2. Some Salmonella biochemical tests (left to right: LD, SIM, Urease, PD, citrate and TSI

typhimurium (all isolates were from the yolk sac). Salmonella samples were collected from two different farms. Based on the history of the fields, Salmonella isolates that were categorized in serotype typhimurium had a high mortality rate within three days of hatching, in contrast, in farms where Salmonella isolates were categorized in ser. Ruzizi, mortality and no signs and symptoms were usual. Eight of 100 considered quail chicks were positive for Enterobacter. Nine Enterobacter isolates included 5 Enterobacter cloacae (1 isolate from the liver and 4 isolates from the yolk sac) and 4 Enterobacter aerogenes (one isolates from the liver and 3 from the volk sac). Some of the biochemical characteristics of enterobacterium are acid-acid reaction without H2S production in TSI medium and IMViC formula negative, negative, positive, positive. It should be noted that LD test was used in addition to other tests in order to separate Enterobacter cloacae (negative) and

Enterobacter aerogenes (positive). Ten quail chicks were positive for Proteus. Overall 12 isolates were collected that included 5 *Proteus vulgaris* (all of them were from yolk sac) and 7 Proteus mirabilis (4 isolates from the liver and 3 from the yolk sac). All the samples were taken from five different farms. Biochemical characteristics of Proteus include alkali-acid reaction with H2S production in TSI medium (Figure 3), urease positive and LD test negative and PD reaction positive. The IMViC formula for *Proteus vulgaris* is positive, positive, negative, negative, and for Proteus mirabilis it is negative, positive, (negative), positive.



Figure 3. Klebsiella colonies in BG medium

Eight quails were positive for Klebsiella pneumonia. The isolates were separated from yolk sac (7 isolates) and liver tissues (3 isolates). Nine of 10 farms in this study were positive for Klebsiella. Some of the biochemical characteristics of Klebsiella consist of acid-acid reaction without H2S production in TSI medium, mucoid colonies in Bordet-Gengou medium (Figure 4), urease positive, immobility in SIM medium, and the IMViC formula negative, (negative), positive, positive. In this study, three quails suffered from mixed infection with Klebsiella and *E. coli*, two quails had synchronous infection of Proteus and *E. coli*, and a co-infection with Klebsiella and Proteus was observed in two quails.

Antibacterial drug susceptibility results. The results of bacterial isolates (Salmonella spp., *E. coli*, Klebsiella, Proteus and Enterobacter) sensitivity to all the 12 antibiotics (i.e., cephalexin, ciprofloxacin, florphenicol, gentamycin, lincospectin, soltrim,

280

phosphomycin, cefotaxime, ceftriaxone, enrofloxacin, doxycycline and oxytetracycline) are shown in Table 2.



Figure 4. Proteus in the TSI medium

Salmonella showed 100% susceptibility to gentamycin, soltrim, oxytetracycline, phosphomycin, florphenicol and cephalexin, 91% susceptibility to doxycycline and ceftriaxone, 36.5% susceptibility to cefotaxime, lincospectin and ciprofloxacin, and 27.5% susceptibility to enrofloxacin. 100% of E. coli isolates showed susceptibility to ceftriaxone. Sensitivity to cephalexin, ciprofloxacin, florphenicol, gentamycin, lincospectin, soltrim, phosphomycin, cefotaxime, enrofloxacin, doxycycline and oxytetracycline was 63.8%, 66%, 40.5%, 78.5%, 49%, 85%, 95.7%, 32%, 24% and 25.5%, respectively. Further, 100% of Klebsiella isolates showed susceptibility to ceftriaxone. Drug susceptibility to cephalexin, ciprofloxacin, florphenicol, gentamycin, lincospectin, soltrim, phosphomycin, cefotaxime, enrofloxacin, doxycycline and oxytetracycline was 61.5%, 69.5%, 38.5%, 61.5%, 46.5%, 92.4%, 92.4%, 61.5%, 46.5%, 30.1% and 69.2%, respectively. Finally, 100% of Proteus isolates were susceptible to phosphomycin. Drug susceptibility to cephalexin, ciprofloxacin, florphenicol, gentamycin, lincospectin, soltrim, cefotaxime, enrofloxacin. doxycycline, oxytetracycline and ceftriaxone was 44.5%, 66.5%, 55.5%, 89%, 44.5%, 44.5%, 78%, 66.5%, 66.5%, 55.5% and 78%, respectively. As regards Enterobacter isolates, 100% of the isolates were susceptible to soltrim and ceftriaxone. Drug resistance to lincospectin and oxytetracycline was 100% (Table 2).

DISCUSSION

Proper hygiene keeps quail mortality at a low level in all stages of growth. One of the most important causes of early mortality in quail chicks is bacterial infection. In this study, the results of bacterial contamination in the yolk sac and liver designated that Enterobacteriaceae are the main cause of yolk sac infection in quail chicks (78%). A 15.8% contamination with the members of Enterobacteriaceae in unhatched quail eggs was reported by Jahantigh and Nili (2010). Contamination of egg shells leads to bacterial penetration through membranes and infection of the developing fetus (Roy et al., 2006). In the present study, the role of Enterobacteriaceae in diseased quail chicks was identified and most bacterial isolates were Escherichia coli (44%), Salmonella ser. ruzizi (5%), Salmonella ser. typhimurium (3%), Proteus vulgaris (5%), Proteus (5%), Klebsiella mirabilis pneumonia (8%). Enterobacter cloacae (4%) and Enterobacter aerogenes (4%). In Jahantigh et al. (2013) study, the most isolated bacteria were E. coli (6.57 %) followed by Salmonella and Proteus spp. (2.98 %), Citrobacter spp. (1.49 %), Klebsiella spp. and other members of Enterobacteriaceae (0.89 %). In the research performed by Roy et al. (2006), it was found that E. coli is responsible for high mortality in infant chicks and reduced hatchability. They showed that improving hatchery health and farm management is needed to control infection and antibiotics resistance. Sharada et al. (2009) recovered E. coli from 76.47% of their samples. The recovered isolates of constituted the largest portion of the isolates and this indicates the acute nature of the disease. During this survey, S. ruzizi and S. typhimurium were isolated from commercial quail chicks. The absence of other Salmonella serovars

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Enterobacter		Klebsilella		proteus		salmonella		E.coli		Antibiotic						
S	Ι	R	S	Ι	R	S	Ι	R	S	Ι	R	S	Ι	R		
25	0	75	31	0	69	5/66	0	5/33	91	0	9	24	15	61	D	ii. A
S	5/37	0	5/61	5/15	23	89	0	11	100	0	0	5/78	0	5/21	GM	
5/87	5/12	0	4/92	0	6/7	100	0	0	100	0	0	85	2	13	FO	ntibiotic poultry
100	0	0	4/92	0	6/7	5/44	22	5/33	100	0	0	49	2/4	8/46	SLT	ics ry ii
0	25	75	2/69	3/15	3/15	5/55	0	5/44	100	0	5/63	5/25	2/4	3/70	Т	s com industry
5/37	5/37	25	5/46	23	5/30	5/66	0	5/33	5/27	9	0	32	5/40	5/27	NFX	stry
5/12	5/12	75	5/38	5/38	23	5/55	11	5/33	100	0	5/54	5/40	34	5/25	FF	common ıstry
0	25	75	5/46	46	5/7	5/44	0	5/55	5/36	9	0	6/40	2/21	2/28	LST	'n
5/12	5/12	75	5/61	31	5/7	5/44	2/22	3/33	100	0	0	8/63	34	2/2	CN	е т
5/12	75	5/12	5/61	23	5/15	78	22	0	5/36	5/63	0	7/95	3/4	0	CTX	ntib
100	0	0	100	0	0	78	22	0	91	9	0	100	0	0	CRO	Humans antibiotics
25	5/62	5/12	69	31	0	5/66	11	5/22	5/36	5/54	9	66	6/10	4/23	СР	S

Table 2. Antibiotic susceptibility pattern of Isolated bacteria from dead quail chicks

S: Sensitive: I: Intermediate: R: Resistant

D: Doxycycline [§] GM: Gentamycin [§] FO: Phosphomycin [§] SLT: Soltrim [§] T: Oxytetracycline [§] NFX: Enrofloxacin [§] FF: Florphenicol [§] LST: Lincospectin [§] CN: Cephalexin [§] CTX: Cefotaxime [§] CRO: Ceftriaxone ⁽CP: Ciprofloxacin [§])

and occurrence of S. typhimurium and ruzizi in these quail chicks may reveal either geographic or temporal variances in Salmonella colonization of birds. Isolation of Salmonella serotype typhimurium from quail carcasses has major public health consequences for consumers (Center for Disease Control and Prevention, 1999). Some researchers reported that the most Salmonella common poultry serovars are S. Heidelberg, S. typhimurium and S. Hadar (Jones et al., 1991; Caldwell et al., 1995; Byrd et al., 1999; Sander et al., 2001). Isolation of S. typhimurium has also been reported from wild and domesticated quails (Helm et al., 1999; Hudson et al., 2000). The nature of Salmonella serovars in poultry in some developing countries was evaluated by Barbour et al. (2015). They reported that the Salmonella serovars were S. hadar, S. blockley, S. irumu, S. anatum, S. reading, S. virchow, S. schwarzengrund, S. westhampton, S. typhimurium, S. derby, and S. heidelberg in South Africa, S. enteritidis, S. typhimurium, S. infantis, S. kentucky, S. tsevie, and S. chiredzi in Egypt, S. kentucky, S. typhimurium, S. paratyphi S. kentucky and S. typhimurium in Indonesia, S. bareilly, S. enteritidis, S. typhimurium, S. paratyphi B, S. cerro, S. mbandaka, S. molade, S. kottbus, and S. gallinarum in India, and S. enteritidis, S. typhimurium, S. i S. derby, S. colindale, S. rissen, S. ruzizi, S. virchow, S. bbrandenburg, S. bredeney, S. muenchen,

S. kortrijk, and S. calabar in Romania. Because of the lack of routine control of Salmonella serovars in developing countries, the incidence of diseases caused by the consumption of Salmonella-contaminated poultry products is expected. Tirziu et al. (2015) isolated eight serotypes of Salmonella enterica sub sp. enterica: Infantis, Bredeney, Virchow, Djugu, Grampian, Brandenburg, Derby and Ruzizi from raw chicken meat. As already mentioned, the rate of Proteus infection in this study was 10.22%. The infection with these bacteria is associated with increased mortality and decreased hatchability (Fyrouz et al., 2011), but its direct role in death of chicks is not clear. The importance of these bacteria is related to the explosion of eggs due to high gas production causing severe pollution and contamination of other eggs in the hatcheries. The widespread use of antibiotics in poultry has a long history in both treatment and growth promotion. Treatment with antibiotics is an important tool in reducing the incidence and mortality from infectious poultry diseases (Freed et al., 1993). Misuse of antibiotics in poultry industry is responsible for the emergence of antibiotic-resistant bacterial strains (Bower and Daeschel, 1999). E. coli is sensitive to many antibiotics. However, E. coli isolated from poultry are often resistant to one or more antibiotics, in particular if they have been used widely in the poultry industry for a long time (e.g., tetracycline) (Allan et al., 1993). E. coli isolates recovered in this survey showed high resistance to enrofloxacin (68%), doxycycline (76%) and oxytetracycline (74.5%). All the E. coli isolates from Roy et al. (2006) study showed high resistance to ampicillin/cloxacillin, chloramphenicol, tetracycline, and cotrimoxazole (100%). They observed the highest sensitivity to nitrofurantoin. All the E. coli isolates in the present study showed susceptibility to ceftriaxone. In other studies, chicken-origin E. coli isolates showed 94% resistance to tetracycline and 100% resistance to tetracycline-ampicillin (Nazer, 1980; Rafiei Tabatabaei and Nasirian, 2003). Paratyphoid infection treatment with antibiotics in poultry is considered. Salmonella serovars obtained in this study displayed several different levels of sensitivity and resistance to different antibiotics. They showed high resistance to cefotaxime, lincospectin and ciprofloxacin (75.5 %) and no resistance against gentamycin, soltrim, oxytetracycline, phosphomycin, florphenicol and cephalexin. Salmonellae isolates from Jahantigh et al. (2013) survey showed more resistance to ampicillin, nalidixic acid and tetracycline (80%). No resistance to gentamicin, furazolidone and norfloxacin was observed. The high level of resistance to antibiotics can result from uncontrolled use of these agents in the treatment of bacterial infections. In Jahantigh and Nili (2010) investigation, pigeon egg-origin Salmonella isolates presented resistance to tetracycline (50%), ampicillin, cephalexin and furazolidone (25%). No resistance was observed against colistin, ciprofloxacin, chloramphenicol, gentamycin, nalidixic and norfloxacin. The antibiotic resistance of Salmonella species with animal and human origins is alarming and has been studied by several authors (Nazer and Safari, 1994; van Duijkeren et al., 2003; Adesiyun et al., 2006; Graziani et al., 2008; Pan et al., 2009; Kasimoglu Dogru et al., 2010). Differences in farm management (i.e., hygiene and use of various antibiotics), geolocation, and method of testing can justify the differences in the antibiotic susceptibility results of the isolated bacteria in the present study and those obtained by the mentioned studies, especially in Iran.

In conclusion, the results of this study suggest that increasing antibiotic resistance has become a major challenge in Iranian poultry industry. The emergence and spread of antibiotic-resistant bacteria in recent years has become a major concern for public health. The reality is that food contamination with antibioticresistant bacteria can be a serious threat to human health, because it is known that most of the antibiotic resistance genes are on plasmids and could be transferred to human pathogenic bacteria (van Duijkeren et al., 2003). High resistance to some of the most widely used drugs in Iranian poultry industry such doxycycline, enrofloxacin, florfenicol as and trimethoprim / sulfadiazine makes the selection of an appropriate and effective antibiotic in the treatment of bacterial infections difficult. For control and treatment of various diseases caused by Enterobacteriaceae in poultry, antibiotic sensitivity testing should be performed and the appropriate antibiotics must be used at a sufficient dose and duration.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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