Research Report

Evaluation of the Acute Toxicity, Irritation, Hypersensitivity and Genetic Toxicity for Lipopolysaccharides from *Pantoea agglomerans* (LPSp)

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DOI: 10.3969/gab.030.000175

Abstract In this study, a serial of experiments were conducted to evaluate the safety of *Pantoea agglomerans* lipopolysaccharide (LPSp). Using the limited does method, the acute oral toxicity test was carried out on Kunming mice to understand acute toxicity of LPSp; the skin and eye Irritation test were carried out on New Zealand Rabbits to understand the skin and eye irritation of LPSp; and the skin allergy test were carried out on guinea pigs to understand the hypersensitivity of LPSp. The salmonella typhimurium/reverse mutation assay and cell micronucleus test was conducted to understand the genetic toxicity of LPSp. The result of the acute oral toxicity test shows that LPSp has no toxicity when LD_{50}>5 000 mg/kg. The skin and eye irritation test shows LPSp has no irritation reaction and hypersensitivity for skin and eye (the integration of both skin and eye are 0). The skin allergy test shows LPSp is not the sensitinogen for guinea pig (both integration and ratio of skin reaction are 0). The salmonella typhimurium/reverse mutation assay was negative (P>0.05). The cell micronucleus test was negative (P>0.05 when compared with negative control, P<0.01 when compared with positive control). It is concluded that LPSp is very safe for these animals in our experiments. It has no toxicity, no irritation, no hypersensitivity and no genetic toxicity.

Keywords *Pantoea agglomerans*, LPS, Toxicity test, Reverse mutation assay, Micronucleus test

Funded by: Guangxi Natural Science Fund (GuiKeQing 0135012) and Guangxi Medical University Doctoral Fund (2004)

Lipopolysaccharide was first known from the study of endotoxin. In 1965, endotoxin was proved to be a kind of biomacromolecules on the outer membrane of Gram-negative bacteria, and was named lipopolysaccharide, LPS. Lipopolysaccharide is mainly composed of 3 parts: lipid A, core polysaccharide and specific polysaccharide. Lipid A is the main part of the outer membrane, and it is also the biological active component of lipopolysaccharide. Literature shows that the lipid A group of LPS molecules is the real toxic component of endotoxin (Galanos et al., 1985). The structures of lipid A synthesized by different bacteria are also different (Caroff and Karibian, 2003). The structure of lipid A plays an important role to the biological activity of bacteria (Montminy et al., 2006). With more researches on the chemical composition, toxic effect and biological activity of lipopolysaccharide, it is found that lipopolysaccharide is toxic and also causes strong immune response. Lipopolysaccharide can activate the mononuclear phagocyte system and elicit the release of cytokines, such as IL-1, IL-6, TNF, IFN, and CSF (Carswell et al., 1975; Lukasiewicz and Lugowski, 2003). In addition, bacterial lipopolysaccharide can induce differentiation and maturation of dendritic cells, and enhance antigen presentation (Granucci et al., 1999). It can also promote complement activation and antibody production, and regulate specific immune (*note by the translators:* it should be *adapted immune*) response (D'Andrea et al., 1993).

Though lipopolysaccharide has great potential for immune regulation (Beutler and Rietschel, 2003), its application in medical care is restricted due to its toxicity and pyrogenicity. Continuous exploration has been conducted on how to remove its toxicity but still retain its immune regulatory activity. Studies in recent years mainly include research on derivatives of lipid A-monophosphoryl lipid A (MPL). GlaxoSmithKline Company developed several new adjuvant systems containing MPL with strong adjuvant effect. AS04 and AS03 were approved in the European Union in 2005 and 2008 respectively (Garcon et al., 2007; de Gregorio et al., 2008), and the toxicity of MPL is only 1% of lipopolysaccharide in preclinical animal experiments (Evans et al., 2003). It shows that lipopolysaccharide can be developed and applied in clinics, and serve human health (Casella and Mitchell, 2008). The *Pantoea agglomerans* lipopolysaccharide, LPSp, in this study is from bacterial lipopolysaccharide of Gram-negative non-pathogenic bacteria *Pantoea agglomerans*, P.A, in wheat flour. The results show that LPSp has many biological effects, such as anti-tumor activity (Iwamoto et al., 1996, Goto et al., 1996), promoting the healing of burnt wounds (Liang Ziqian et al., 2000), enhancing immune functions (Huang Zhiming et al., 2002), and that it has great adjuvant effects (Leng et al., 2004; Wang Jian et al., 2007). These results point out that LPSp has great effect on medical care, and it is also a good biological immune regulator. It has potential in practical application and development. According to the related evaluation criteria and methods from *Hygienic Standards for Cosmetics (2007)* and *Technical Standards for Testing and Evaluating of Supplement Food (2003)* by the Ministry of Health of the People's Republic of China, this paper conducts a preliminary research on the safety of LPSp. It provides the experimental safety basis for the development and application of LPSp.

1 Results and analysis

1.1 Acute toxicity test of LPSp

After treatment, there was no abnormality in the general condition and behavior of the mice during the observation period. The mice gained weight and there was no mice death. After gross anatomy, there was no abnormal change in viscera, such as heart, lung, liver, kidney, spleen, stomach and intestine. According to the acute toxicity grading standard, the test with healthy adult mice of empty stomach through single gavage shows LPSp is considered non-toxic at LD_{50}>5 000 mg/kg (see Table 1).

Table 1 The acute oral toxicity test results of LPSp in mice

Gender	Dose (mg/kg)	Number of mice	Weight (x±s)(g) 0d	7d	14d	Number of death mice	Death rate (%)	LD_{50} (mg/kg)
Female	5000	10	20.6±1.5	24.1±1.9	27.7±1.8	0	0	> 5000
Male	5000	10	20.9±1.3	25.2±1.4	28.9±1.5	0	0	> 5000

1.2 Acute dermal irritation test

After single application of LPSp on mice skin, there was no erythema and edema on the tested area. The average total score of the dermal irritation test is 0. According to the skin stimulate intensity standard, LPSp causes no acute skin irritation (see Table 2).

Table 2 The acute dermal irritation test results of LPSp on rabbits

Coding No.	Weight(kg)	1h		24h		48h		72h	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control
		A B C	A B C	A B C	A B C	A B C	A B C	A B C	A B C
1	2.2	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
2	2.0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
3	2.5	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
4	2.4	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Average of total score		0	0	0	0	0	0	0	0
Stimulate Intensity grading		Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant

Note: A: Erythema; B: Edema; C: Total score

1.3 Multiple dermal irritation test

After application of LPSp on mice skin for 14 consecutive days, there was no irritation, such as erythema and edema, on the tested area. The average daily score of each rabbit is 0. According to the skin stimulation intensity standard, LPSp causes no apparent skin irritation in the experiment (Table 3).

Table 3 The multiple dermal irritation test results of LPSp on rabbit

Application Days (d)	Number of rabbits	Irritation reaction score					
		Sample			Control		
		Erythema	Edema	Total score	Erythema	Edema	Total score
1	4	0	0	0	0	0	0
2	4	0	0	0	0	0	0
3	4	0	0	0	0	0	0
4	4	0	0	0	0	0	0

5	4	0	0	0	0	0	0
6	4	0	0	0	0	0	0
7	4	0	0	0	0	0	0
8	4	0	0	0	0	0	0
9	4	0	0	0	0	0	0
10	4	0	0	0	0	0	0
11	4	0	0	0	0	0	0
12	4	0	0	0	0	0	0
13	4	0	0	0	0	0	0
14	4	0	0	0	0	0	0
Average of each rabbit score of 14 days		0	0	0	0	0	0
Average of each rabbit score of everyday		0	0	0	0	0	0
Stimulation intensity grading		Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant

1.4 Acute eye irritation test

After one treatment, damage to rabbit's cornea, iris and conjunctiva was evaluated at different time points. Both the average and the maximum scores of irritation reaction are 0. According to the eye irritation standard, LPSp causes no acute eye irritation (see Table 4).

Table 4 The acute eye irritation test results of LPSp on rabbits

Coding No.	Region	Eye irritation reaction score							
		1h		24h		48h		72h	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control
1	Conjunctiva	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0
	Cornea	0	0	0	0	0	0	0	0
2	Conjunctiva	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0
	Cornea	0	0	0	0	0	0	0	0
3	Conjunctiva	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0
	Cornea	0	0	0	0	0	0	0	0
Average of irritation reaction score		0	0	0	0	0	0	0	0
Highest score of irritation reaction score		0	0	0	0	0	0	0	0
Irritation reaction grading		Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant	Nonirritant

1.5 Skin sensitization test

After a boosting treatment, there was no erythema and edema on guinea pigs in both LPSp and distilled water groups during the observation period. The score of skin reaction at all time points is 0, and its sensitization rate is 0%. According to the sensitization intensity standard, LPSp causes no sensitization. In contrast, the positive control group showed mild to severe erythema and edema in the tested area after a boost. The sensitization rate is 100% (Table 5).

Table 5 The skin sensitization test results of LPSp on guinea pigs

Group	Number of mice	Induction dose (mL per mice)	Booster dose (mL per mice)	Average score of skin allergy reaction 24h	48h	Sensitization rate(%)	Sensitization strength assessment
Distilled water group	10	0.2	0.2	0	0	0	Not found
Positive control group	10	0.2	0.2	2.7	2.1	100	Very strong
LPSp group	20	0.2	0.2	0	0	0	Not found

1.6 Salmonella typhimurium/reverse mutation assay

As shown in Table 6, the number of colonies with reverse mutation in all LPSp groups at various dosage did not exceed twice the number in the control group. There was no statistical significance ($P>0.05$) by *t* test. Its mutagenicity test was negative while the mutagenicity test of positive control groups was positive ($P<0.01$). The results show that under these experimental conditions, LPSp has no genetic mutation effect on the 4 tested salmonella typhimurium histidine deficient strains of TA97, TA98, TA100 and TA102 in the presence or absence of S-9.

Table 6 Salmonella typhimurium/reverse mutation assay results of LPSp (x±s)

Group	Dosage	TA97		TA98		TA100		TA102	
	(mg/plate)	-(S-9)	+(S-9)	-(S-9)	+(S-9)	-(S-9)	+(S-9)	-(S-9)	+(S-9)
LPSp	100	170.0±8.18	167.0±7.79	42.0±9.91	44.0±10.11	160.0±3.82	165.0±7.06	197.0±10.11	207.0±39.97
	50	170.0±5.47	168.0±7.67	38.0±7.71	41.0±5.64	167.0±7.03	161.0±8.93	191.0±12.56	196.0±7.68
	25	166.0±7.12	173.0±6.02	40.0±11.54	40.0±6.86	170.0±8.98	165.0±6.75	201.0±10.30	205.0±34.20
	12.5	173.0±5.31	171.0±6.72	37.0±6.83	41.0±4.32	166.0±7.66	166.0±4.83	193.0±10.58	195.0±8.80
	6.25	164.0±4.83	169.0±7.28	39.0±6.93	38.0±3.79	172.0±7.71	168.0±13.39	201.0±11.57	198.0±11.53
	0.1	174.0±5.32	177.0±12.47	42.0±6.69	37.0±4.93	165.0±9.91	167.0±11.30	204.0±34.93	207.0±20.03
Control (water)									
2-AF	10		1626.0± 347.56[a]		1193.0± 325.17[a]		1559.0± 486.67[a]		1940.0± 87.18[a]
NaN3	1.5					671.0± 204.93[a]			
MMC	0.5							839.0± 145.06[a]	
DNR	5	940.0± 101.49[a]		556.0± 108.05[a]					

Note: Compared with the solvent control group, [a]$P<0.01$

1.7 Cell micronucleus test of the mouse bone marrow

Statistical analysis of the test results shows the LPSp micronucleus rate with various dosage has no difference compared with the negative control group ($P>0.05$), and there is

no dosage-reaction relation. However, the positive control group is significantly higher than the negative control group ($P<0.01$) (see Table 7). The results show that under these experimental conditions, LPSp does not increase the micronucleus rate of mammal bone marrow polychromatic erythrocyte (PCE). The micronucleus test was negative.

Table 7 Effects of LPSp on the micronucleus rate in polychromatic erythrocytes of the mouse bone marrow

Group	Gender	No. of mice	PCE No. of checked	Contain micro nuclear cell count	Micro nuclear rate‰
High dosage group	Male	5	5000	8	1.6
	Female	5	5000	9	1.8
Medium dosage group	Male	5	5000	4	0.8
	Female	5	5000	7	1.4
Low dosage group	Male	5	5000	5	1.0
	Female	5	5000	11	2.2
Lowest dosage group	Male	5	5000	7	1.4
	Female	5	5000	9	1.8
Negative control group	Male	5	5000	9	1.8
	Female	5	5000	8	1.6
Positive control group	Male	5	5000	131	26.2[a]
	Female	5	5000	106	21.2[a]

Note: Compared with the negative control group, [a]$P<0.01$

2. Discussion

LPSp in this study was from bacterial lipopolysaccharide of Gram-negative non-pathogenic bacteria *Pantoea agglomerans*, P.A, in wheat flour. Compared with other bacteria LPS, LPSp in this study has low relative molecular mass (about 5000), high activity, and high KDO (3-deoxy -D-mannose–octulose). Its basic structure is also different from other LPS. LPSp has been found to have at least 2 lipid A structures, one being similar to lipid A of *E. coli* LPS and the other similar to lipid A of salmonella Minnesota. The biological activity of LPSp is higher than that of LPS of a single lipid A structure. LPSp is more capable in activating macrophages to produce TNF than LPS of *E. coli*, and its structure is less susceptible to degradation *in vivo* (Leng Jing et al., 2003). It has positive effect on various diseases, such as tumor, ulcer, burn and herpes (Iwamoto et al., 1996). In addition, intradermal injection of LPSp to tumor patients showed antitumor result with no side effects (Goto et al., 1996). Animal experiments showed that LPSp can enhance immunity significantly (Huang Zhiming et al., 2002). LPSp has good adjuvant effect on HBsAg of Hepatitis B virus through activation of macrophages by enhancing antigen processing and presentation (Leng et al., 2004; Wang Jian et al., 2007). Therefore, the safety of LPSp has been evaluated in this study using the acute oral

toxicity test, skin irritation test, skin allergy test, salmonella typhimurium/reverse mutation assay and cell micronucleus test. This provides preliminary safety evaluation criteria for LPSp application.

To understand the acute toxicity of LPSp, the acute oral toxicity test was carried out. *Hygienic Standard for Cosmetics* and *Technical Standard for Testing and Evaluating of Functional Food* in China stipulates that maximum limits can be used when the toxicity of tested samples is very low. Therefore, 10 animals (half male and half female) were treated with 5000mg/kg orally. There was no animal death. It can be considered that the acute oral toxicity test at various dosages is no longer needed. Maximum limits were used in this study. The results show that LPSp at $LD_{50} > 5\ 000$ mg/kg is very safe with no toxicity though its long term toxicity needs further study.

To understand the effect of LPSp on skin, mucous membrane and immune systems, the skin and eye irritation test and skin allergy test were carried out. The results show that there was no erythema and edema on the tested areas on rabbits, and no damage on cornea, iris and conjunctiva. This indicates that LPSp has no significant skin irritation and no acute eye irritation on rabbits. The skin sensitization test could evaluate whether repeated treatments cause allergy reaction to mammals and its severity. The results show that LPSp causes no hypersensitivity to tested animals.

In addition, the salmonella typhimurium/reverse mutation assay and cell micronucleus test of mouse bone marrow were carried out to study the mutagenicity induced by LPSp. Salmonella typhimurium/reverse mutation assays can evaluate the changes in gene sequences. Cell micronucleus were carried out to measure chromosomal mutation induced by clastogen and some aneuploidy-inducing agents. The mutagenicity was analyzed and the possibility of genetic damage and dormant carcinogenic effect were predicted. This was conducted using test materials from microorganism and the somatic cells of mammals in vitro and in vivo. The salmonella typhimurium/reverse mutation assay results of LPSp on tested strains shows that no mutagenic effect was found. Under these experimental conditions, no mutagenic effect of LPSp was observed in the cell micronucleus test. It indicates that LPSp has no genetic damage and dormant carcinogenicity.

It is concluded that LPSp is an immune regulator which merits further study, development and application. It is very safe in the experiments with no significant irritation on skin and eye, no hypersensitivity, no genetic damage and dormant carcinogenicity,

3. Materials and methods

3.1 Materials

Kunming mice, specific-pathogen free, weighing 18~22g; New Zealand rabbits, non-specific-pathogen free, weighing 2000~2500g; guinea pigs, non- specific-pathogen free, weighing250~300g. They were purchased from the Experimental Animal Center, Guangxi Medical University (License No. GuiDongXuZi 2000 No. 1). The 4 standard test salmonella typhimurium histidine deficient strains of TA97, TA98, TA100 and

TA102 were provided by the Center for Disease Control and Prevention in Guangxi District. The strains were tested before the experiment and were preserved at -80℃ for future use. LPSp was prepared with sterile distilled water and preserved by the Immunology Teaching and Research Unit at Guangxi Medical University. 2,4-Dinitrochlorobenzene was from No.1 Factory Of Shanghai Reagents with batch number of 20040801. It was prepared with acetone into 1% induce concentration and 0.1% provoking concentration before use.

3.2 Acute toxicity test of LPSp

Ten healthy adult Kunming mice, half male and half female, weighing 18~22g, water but no food overnight before the test. The limited dose method was adopted. That is, the 10 mice were treated 5000mg/kg through one gavave. The dose of LPSp was given as 20mL/kg bw. After treatment, the mice were observed for 14 consecutive days while recording their general condition, toxic symptoms and death status everyday and weighing surviving animals every week during the observation period. After observation, weigh and euthanize the mice and then conduct gross anatomy. Record the changes of the viscera and grade the acute toxicity according to the results.

3.3 Acute dermal irritation test

Four healthy adult female New Zealand rabbits, weighing 2000~2500 g. Hair from both two sides of rabbits' dorsal spine was removed in an area of about 3 cm×3 cm each side 24 hours before the test. Directly apply 0.5mL 1g/mL LPSp prepared with sterile distilled water over one side of sheared skin in an area of 2.5 cm×2.5 cm. Then cover with two layers of gauze and one layer of cellophane, and then fix with non-irritating adhesive plaster and bandage. Apply terile distilled water over the other side as comparison. Each rabbit was put in a separate cage. After 4 hours, wipe out the residual test materials on the skin with warm water. Observe and record the reaction of the treated skin area after 1 hour, 4 hours, 48 hours and 72 hours respectively, and then score the skin irritation reaction and grade the skin stimulation intensity.

3.4 Multiple dermal irritation test

Four healthy adult female New Zealand rabbits were treated in the same was as in the above acute dermal irritation test. Apply once a day for 14 days. From the 2^{nd} day, remove hair before applying every time, and wipe out the residual test material on the skin with warm water. Do the same for both the control and test areas. Observe and record the reaction of the applied skin area after 1 hour and score the skin irritation reaction. After the experiment, calculate the average score for each rabbit everyday according to the formula: (Σerythema and edema score/number of tested animals)/14, and then evaluate the skin stimulation intensity.

3.5 Acute eye irritation test

Three healthy adult New Zealand rabbits were used in this test. Gently open the lower eyelid of one rabbit eye, drip 0.1mL test material (containing 100mg LPSp) into the conjunctival sac, keep the upper and lower eyelids closed for 1second, and do not wash for 24 hours. The other eye was used as the control without any treatment. Check the eyes 1h, 24h, 48h and 72 h after treatment, observe and record local eye irritation reaction, and

grade the eye irritation intensity.

3.6 Skin sensitization test

Divide 40 healthy guinea pigs with weight between 250~300 g into the test group (1 g/mL LPSp) of 20 guinea pigs, the negative control group (sterile distilled water) of 10 guinea pigs and the positive control group (1% 2,4- Dinitrochlorobenzene) of 10, half male and half female in each group. Remove hair in an area of about 3 cm×3 cm on the left side of each guinea pig's back 24 hours before the test. Apply 0.2mL LPSp on the sheared area, and cover it with two layers of gauze and one layer of cellophane, fix with non-irritating adhesive plaster for 6 hours, and then wipe out the residual test material on the skin with warm water. Repeat the treatment on the 7^{th} and 14^{th} day in order to contact-induce. Treat the negative and positive control groups the same way as for the test group. That is, treat the two groups with sterile distilled water and 1% 2,4-Dinitrochlorobenzene respectively in order to contact-induce. At the 14th day after the last induction, apply 0.2mL 1g/mL LPSp to the test and the negative control groups and 0.2mL 0.1% 2,4- Dinitrochlorobenzen to the positive control group, on guinea pigs' right side (remove hair 24 hours in advance). Likewise, cover and fix for 6 hours, and then wipe out the test material. Then observe local skin reaction 24h and 48h afterwards and score the skin allergy reaction. Calculate the sensitization rate and the average score of skin allergy reaction according to the following formula, and measure the sensitization intesivity. Sensitization rate (%)=Number of animals with allergy reaction /Total number of animals ×100%; Average score of skin allergy reaction = Total score of all animals / Total number of animals.

3.7 Salmonella typhimurium/reverse mutation assay

A group of standard test strains of TA97, TA98, TA100 and TA102 was used and tested for biological characteristics. LPSp were evaluated using the plate incorporation method in the presence or absence of S-9. In this assay, LPSp was divided in 5 dose groups: 100 mg/plate, 50 mg/plate, 25 mg/plate, 12.5 mg/plate and 6.25 mg/plate. Each reagnet was prepared with sterile distilled water. The negative control group was prepared with solvent (sterile distilled water). The positive control groups were made up by 2-AF, NaN3, MMC, and DNR. Three plates were prepared for each reagent. In the top layer of agar, add 0.1mL test strain enrichment broth and 0.1mL test solution. Add 0.5mL S-9 mixture during metabolic activation. Mix evenly and rapidly pour into the bottom layer of agar. After the solidification of petri dish, put into a 37℃ incubator for 48 hours, and record the number of colonies with reverse mutation. The results show that if the number of colonies with reverse mutation in the test group is twice or more than that of the solvent control group and if statistical analysis indicates a dosage-reverse mutation reaction relation and repeatability, then the test materials were considered mutation test positive.

3.8 Cell micronucleus test of mouse bone marrow

Divide test animals randomly into 4 sample groups, and 6 negative control groups (sterile distilled water) and 6 positive groups (40 mg/kg cyclophosphamide),10 mice in each group, half male and half female. The dosage for the 4 sample groups was 40.0 mg/kg, 20.0 mg/kg, 10.0 mg/kg and 5.0 mg/kg respectively. Treat the test animals LPSp twice by

gavage in 30 hours. That is, treat the tested animals the 2nd time 24 hours after the first treatment, euthanize after 6 hours, make slides of sternum bone marrow, fix, stain and obtain cell count. Count 1,000 polychromatic erythrocytes of each animal, and calculate the micro nuclear rate.

3.9 Statistical processing method

Statistical analysis was conducted using the statistical software SPSS 13.0. The data of salmonella typhimurium/reverse mutation assay was processed using *t* test. The results were represented by x±s, and the micro nuclear rate was tested by χ^2.

Author contributions

Wang Qihui and Shen Jiqing conducted the experiments in this study. Wei Jinbin participated in experimental guidance, discussion, paper writing and revisison. Zeng Xia participated in experimental guidance, paper writing and revision. Wang Jian participated in experimental design and analysis of test results. Leng Jing was the project manager, who gave guidance in experimental design, data analysis, paper writing and revision.

Acknowledgements

Our acknowledgement goes deeply to Professor Soma Genichiro at Tokushima Bunri University of Japan for his technological guidance on LPSp preparation. We would also like to thank the two anonymous peers and the editor for their peer review feedback and revision suggestions.

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