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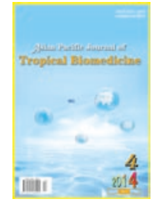

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Comparative susceptibility to permethrin of two *Anopheles gambiae s.l.* populations from Southern Benin, regarding mosquito sex, physiological status, and mosquito age

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PEER REVIEW

Peer reviewer

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Comments

This is a good work in which the authors tried to determine the effect of some factors such as sex, age and physiological status of mosquito specimens on the results of the WHO standard insecticide susceptibility tests. The results could be useful for considering these and other probable effective factors during the WHO standard susceptibility tests.
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ABSTRACT

Objective: To investigate what kind of mosquito sample is necessary for the determination of insecticide susceptibility in malaria vectors.
Methods: Larvae and pupae of *Anopheles gambiae s.l.* (*An. gambiae*) mosquitoes were collected from the breeding sites in Littoral and Oueme departments. The Centers for Disease Control and Prevention (CDC) susceptibility tests were conducted on unfed male and female mosquitoes aged 2-5 days old. CDC susceptibility tests were also conducted on unfed, blood fed and gravid female mosquitoes aged 2-5 days old. These susceptibility tests were also conducted on unfed and blood fed female mosquitoes aged 2-5 days old and 20 days old. CDC biochemical assay using synergist was also carried out to detect any increase in the activity of enzyme typically involved in insecticide metabolism.
Results: Female *An. gambiae* Ladji and Sekandji populations were more susceptible than the males when they were unfed and aged 2-5 days old. The mortality rates of blood fed female *An. gambiae* Ladji and Sekandji populations aged 2-5 days old were lower than those obtained when females were unfed. In addition, the mortality rates of gravid female *An. gambiae* Ladji and Sekandji populations aged 2-5 days old were lower than those obtained when they were unfed. The mortality rate obtained when female *An. gambiae* Sekandji populations were unfed and aged 20 days old was higher than the one obtained when these populations were unfed and aged 2-5 days old. The results obtained after effects of synergist penicillin in beeswax on F1 progeny of *An. gambiae* Ladji populations resistant to permethrin showed that mono-oxygenases were involved in permethrin resistant F1 progeny from Ladji.
Conclusions: The resistance is a hereditary and dynamic phenomenon which can be due to metabolic mechanisms like overproduction of detoxifying enzymes activity. Many factors influence vector susceptibility to insecticide. Among these factors, there are mosquito sex, mosquito age, its physiological status. Therefore, it is useful to respect the World Health Organization criteria in the assessment of insecticide susceptibility tests in malaria vectors. Otherwise, susceptibility testing is conducted using unfed female mosquitoes aged 3-5 days old. Tests should also be carried out at (25±2) °C and (80±10)% relative humidity.

KEYWORDS
Mosquito sex, Physiological status, Mosquito age, Susceptibility, Permethrin, Synergist

1. Introduction

Malaria is a severe public health problem. In 2009, there were an estimated 225 million cases of malaria all over the world[1]. The vast majority of cases in 2009 (78%) were in the African region, followed by the South-East Asia (15%) and Eastern Mediterranean regions (5%). Most victims are children under five years old living in sub-

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Saharan Africa[1]. Malaria is transmitted by female *Anopheles* mosquitoes, and because there is currently no vaccine available, vector control is one of the most important means of malaria prevention.

The knowledge regarding resistance level to insecticide in malaria vectors remains the first stage in the implementation of vector control strategies.

World Health Organization (WHO) recommended that in the National Malaria Control Programmes of each African country, there was a service in the assessment of susceptibility tests in malaria vectors. WHO also recommended three kinds of mosquito sample in the assessment of these tests. These samples are: 1) adult females derived from larval collections; 2) the F1 progeny of wild-caught female mosquitoes; 3) wild-caught females directly[2].

Female mosquitoes during their blood meals, can take a certain dose of insecticide available on the impregnated materials such as insecticide-treated nets. Thus, it would be useful to compare mosquito susceptibility when they were blood fed to when they were unfed. In Ladji and Sekandji localities, both located in Southern Benin, it was shown that detoxifying enzymes like mono-oxygenases played a role in *Anopheles gambiae* (*An. gambiae*) Ladji and Sekandji populations resistant to permethrin in 2008 and to deltamethrin in 2010 respectively[3]. So, it is important to check if this detoxifying enzymes activity detected in these mosquito populations is also present within their F1 progeny. In fact, when it is difficult to collect a sufficient number of larvae and pupae of *An. gambiae* mosquitoes during the dry seasons of the year, for instance, the scare larvae and pupae collected can be reproduced.

Female F1 progeny from this reproduction can be used in the assessment of susceptibility tests in malaria vectors. Susceptibility tests are usually assessed using unfed female mosquitoes aged 2-5 days old[2]. However, is it possible to use male *An. gambiae* mosquitoes in the assessment of these susceptibility tests when it becomes difficult to respect these criteria required by WHO?

The aim of this study was to compare susceptibility to permethrin of two *An. gambiae s.l.* samples from Southern Benin, regarding mosquito sex, physiological status, and mosquito age.

2. Materials and methods

2.1. Study area

The study was carried out in the south of Benin, more precisely in Ladji, in the Cotonou district of Littoral department and in Sekandji, in the Seme district of Oueme department (Figure 1). The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, the Long-Lasting Insecticidal Nets, OlysetNets distribution recently by National Malaria Control Programme in these localities and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. Cotonou is characterized by a tropical coastal guinean climate with two rainy seasons (April-July and September-November). The mean annual rainfall is over 1 500 mm. Oueme has a climate with two rainy seasons (March-July and September-November). The temperature ranges from 25-30 °C with the annual mean rainfall between 900 and 1 500 mm.

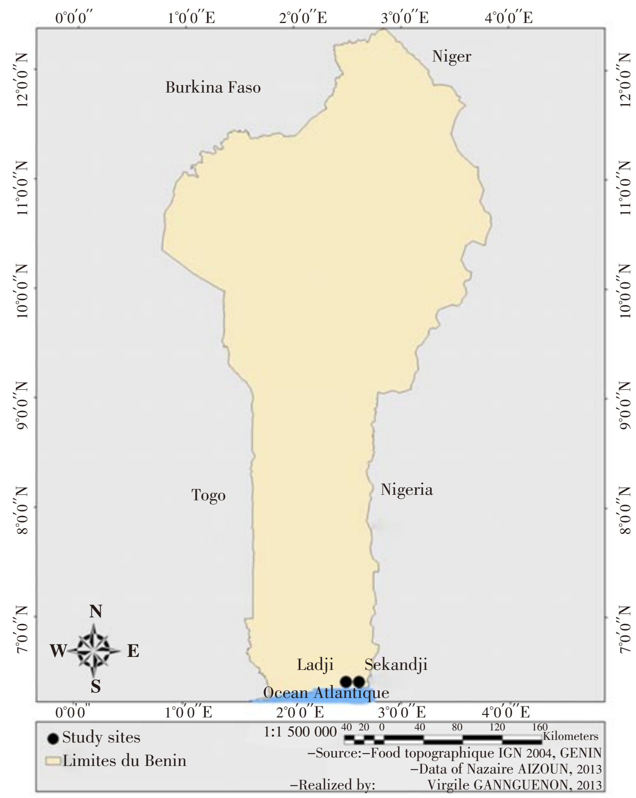

Figure 1. Map of the study area.

2.2. Sample collection

An. gambiae s.l. mosquitoes were collected during the first rainy season (March-July 2013) across Sekandji in the Seme district selected in South Benin and across Ladji in the Cotonou district also selected in South Benin. Larvae and pupae were collected on breeding sites using the dipping method. They were then kept in separated labeled bottles related to each locality. The samples were reared up to adult emergence at the Centre de Recherche Entomologique de Cotonou insectary. *An. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. Susceptibility tests were carried out following Centers for Disease Control and Prevention (CDC) protocols. All susceptibility tests were conducted in the Centre de Recherche Entomologique de Cotonou laboratory at (25±2) °C and 70% to 80% relative humidity.

2.3. Obtaining of F1 progeny

After larvae and pupae were collected in Ladji location, they were reared up to adult emergence at insectary. Male and female adult mosquitoes aged 5–7 days old were used in the reproduction. After the female mosquitoes had been mated and given rabbit's blood meal, an ovipositor was put in mosquito cage containing these females. After the eggs were laid by these females, they were placed in some containers which contained water. Larvae of first stage were fed with yeast. They were then reared up to F1 progeny emergence.

2.4. Obtaining of old female mosquitoes

After larvae and pupae were collected in Ladji and Sekandji locations, they were reared up to adult emergence at insectary. Adult mosquitoes were provided with cotton wool moistened with a 10% honey solution until they were 19 days old. On Day 20, they were separated in two batches. The first batch was fed with rabbit's blood meal and susceptibility tests were assessed the same day on blood fed old female mosquitoes. On this same day (Day 20), the second batch containing unfed old female mosquitoes was also used in the assessment of the susceptibility tests.

2.5. Testing insecticide susceptibility using CDC protocol

The principle of the CDC bottle bioassay is to determine the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relative to a susceptible control. Anything that prevents or delays the compound from achieving its objective of killing the arthropods contributes to resistance. Diagnostic dose that was applied in the present study was the dose recommended by CDC[4]. This dose was checked on the *An. gambiae* Kisumu susceptible reference strain before being applied to field populations. For *An. gambiae s.l.*, the diagnostic dose of 21.5 μg per bottle for permethrin was used for the diagnostic exposure time of 30 min. The choice of permethrin was justified by the insecticide used on OlysetNets that were distributed free by the National Malaria Control Programme in July 2011 across the entire country. The solution was prepared and the bottles coated according to the CDC protocol[4]. A total of 15 to 20 unfed male and female mosquitoes aged 2–5 days old were introduced separately into four 250 mL Wheaton coated bottles with insecticide and one control bottle coated with acetone only. We have also introduced 15 to 20 unfed, blood fed and gravid female mosquitoes aged 2–5 days old separately into four 250 mL Wheaton coated bottles with insecticide and one control bottle coated with acetone only. A total of 15 to 20 unfed and blood fed female mosquitoes aged 2–5 days old and 20 days old were also separately introduced into four 250 mL Wheaton coated bottles with insecticide and one control bottle coated with acetone only. The number of dead or alive mosquitoes was monitored at different time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min). This allowed us to determine the percentage of total mortality (Y axis) against the exposure time (X axis) for all replicates using a linear scale.

2.6. Biochemical assay using synergist

Synergist was used according to the protocol described by CDC[4,5] following the procedure outlined in Figure 2. Samples of F1 progeny that showed high resistance to permethrin in Ladji from the Cotonou district were exposed to the effects of synergist: piperonyl butoxide (PBO) (400 μg per bottle), which inhibits oxidase activity. This synergist was used only with combination with permethrin alone.

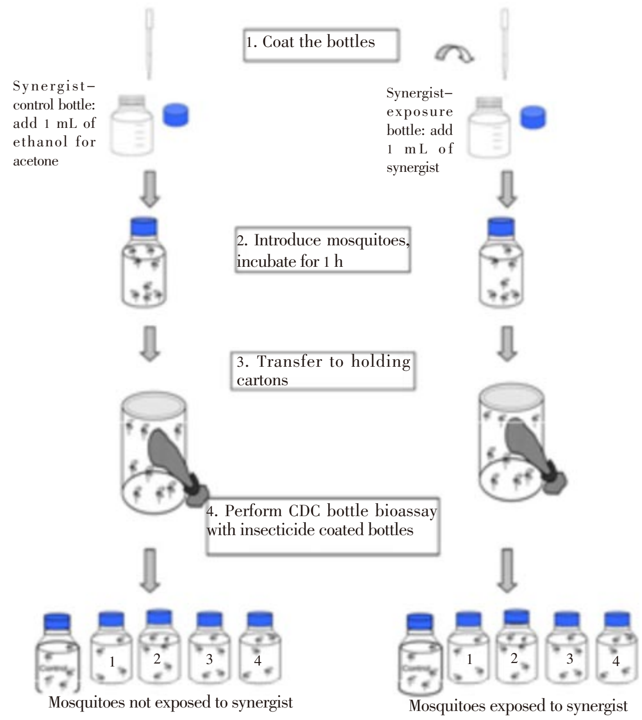

Figure 2. Diagram of performing the CDC bottle bioassay with synergists (CDC guideline, 2010).

Approximately 125 mosquitoes were used for the synergist assay. The number of dead or alive mosquitoes was monitored at different time intervals (0, 15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min). This test allowed us to compare the obtained percentages of dead mosquitoes (Y axis) against time (X axis) before the addition of the synergist to that obtained after the addition of the synergist (Figure 2).

2.7. Statistical analysis

Stata 12 was used to analysis the data sets gathered from the two localities surveyed to compare for the tested insecticide, the mortality rates of *An. gambiae* populations obtained regarding mosquito sex, physiological status and mosquito age. To appreciate the effects of synergist PBO on F1 progeny of *An. gambiae* Ladji populations resistant to permethrin, we used a Kruskal-Wallis test. Data are presented with 95% confidence limits.

3. Results

3.1. Comparison of mosquito susceptibility regarding their sex

The analysis of Table 1 shows that both sexes of *An. gambiae* Kisumu populations were fully susceptible to permethrin when they were unfed and aged 2–5 days old. Regarding *An. gambiae* Ladji and Sekandji populations, females were more susceptible than males when they were unfed and aged 2–5 days old (P<0.05) (Table 1).

Table 1

Comparative susceptibility of *An. gambiae* populations aged 2–5 days old regarding their sex.

Locality	Mosquito sex	Physiological status	Number tested	Mortality (%)
Kisumu	Male	Unfed	94	100.00%
	Female	Unfed	97	100.00%
Ladji	Male	Unfed	64	19.00%
	Female	Unfed	60	88.33%
Sekandji	Male	Unfed	52	28.84%
	Female	Unfed	100	62.00%

3.2. Comparison of mosquito susceptibility regarding their physiological status

The analysis of Table 2 shows on the one hand that female *An. gambiae* Kisumu populations were fully susceptible to permethrin when they were unfed, blood fed, gravid and aged 2–5 days old. The analysis of Table 2 shows on the second hand that the mortality rates of blood fed female *An. gambiae* Ladji and Sekandji populations aged 2–5 days old were lower than those obtained when these females were unfed (P<0.05). Regarding the mortality rate of gravid females from Ladji, it was not significantly different from the one obtained when these females were unfed (P>0.05). However, the mortality rate of gravid females from Sekandji was significantly different from the one obtained when these females were unfed (P<0.05) (Table 2).

Table 2

Comparative susceptibility of *An. gambiae* populations aged 2–5 days old regarding their physiological status.

Locality	Mosquito sex	Physiological status	Number tested	Mortality (%)
Kisumu	Female	Unfed	97	100.00%
	Female	Blood fed	68	100.00%
	Female	Gravid	73	100.00%
Ladji	Female	Unfed	60	88.33%
	Female	Blood fed	77	3.89%
	Female	Gravid	47	80.85%
Sekandji	Female	Unfed	100	62.00%
	Female	Blood fed	115	25.21%
	Female	Gravid	104	33.65%

3.3. Comparison of mosquito susceptibility regarding their age

The analysis of Table 3 shows on the one hand that female *An. gambiae* Kisumu populations were fully susceptible to permethrin when they were unfed, aged 2–5 days old and aged 20 days old. The analysis of Table 3 shows on the second hand that female *An. gambiae* Kisumu populations were fully susceptible to permethrin when they were blood fed aged 2–5 days old and aged 20 days old. The analysis of this table shows that the mortality rates of blood fed female *An. gambiae* Ladji and Sekandji populations aged 20 days old were higher than those obtained when these populations were blood fed and aged 2–5 days old. A similar pattern was observed in female *An. gambiae* Sekandji populations when they were unfed, aged 20 days old comparatively to when they were unfed and aged 2–5 days old. However, the mortality rate obtained when female *An. gambiae* Ladji populations were unfed and aged 20 days old was significantly lower than the one obtained when they were unfed and aged 2–5 days old (P<0.05).

Table 3

Comparative susceptibility of *An. gambiae* populations regarding mosquito age.

Locality	Mosquito sex	Physiological status	Mosquito age	Number tested	Mortality (%)
Kisumu	Female	Unfed	2–5 days old	97	100.00%
	Female	Unfed	20 days old	72	100.00%
	Female	Blood fed	2–5 days old	68	100.00%
	Female	Blood fed	20 days old	57	100.00%
Ladji	Female	Unfed	2–5 days old	60	88.33%
	Female	Unfed	20 days old	47	30.00%
Sekandji	Female	Unfed	2–5 days old	100	62.00%
	Female	Unfed	20 days old	44	93.18%
Ladji	Female	Blood fed	2–5 days old	77	3.89%
	Female	Blood fed	20 days old	19	21.05%
Sekandji	Femelle	Blood fed	2–5 days old	115	25.21%
	Femelle	Blood fed	20 days old	46	56.52%

3.4. Effects of synergist PBO on F1 progeny of An. gambiae Ladji populations resistant to permethrin

The analysis of Figure 3 shows that after the addition of synergist PBO to permethrin 21.5 μg per bottle, the percentage of dead F1 progeny mosquitoes from Ladji is higher than the one obtained with permethrin alone. The use of PBO synergist in bottles treated with permethrin 21.5 μg per bottle did not eliminate permethrin resistance, but significantly reduced the level, in point of fact that the mortality rate increased from 18.36% to 61.33% (P<0.05). These results suggest an implication of mono-oxygenases in resistance of F1 progeny of *An. gambiae s.l.* to pyrethroids.

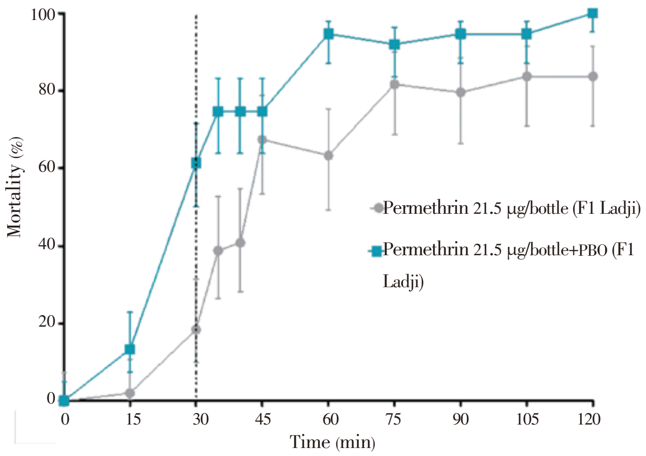

Figure 3. Implication of mono-oxygenases in resistance of F1 progeny of *An. gambiae s.l.* to pyrethroid in Ladji locality.

4. Discussion

Female *An. gambiae* Ladji and Sekandji populations were more susceptible than the males when they were unfed and aged 2-5 days old. Although males are usually smaller and more fragile than females[2], they were not more susceptible than the females. However, it was not recommended to assess susceptibility tests with males because that tends to have higher control mortalities[2,4]. In addition, male *Anopheles* mosquitoes were not able to transmit malaria. For this reason, susceptibility testing is conducted using only female mosquitoes.

The mortality rates of blood fed female *An. gambiae* Ladji and Sekandji populations aged 2-5 days old were lower than those obtained when females were unfed. This result shows that there was no increasing in vector susceptibility to permethrin after females had taken their blood meals. A similar pattern was observed in *Anopheles funestus*[6]. The overproduction of detoxifying enzymes activity could explain why blood fed *An. gambiae* Ladji and Sekandji populations aged 2-5 days old were more resistant to permethrin comparatively to when they were unfed. In addition, the mortality rates of gravid female *An. gambiae* Ladji and Sekandji populations aged 2-5 days old were lower than those obtained when they were unfed. This result confirms that the blood meal really contributed to mosquito tolerance. However, a converse situation was observed in *An. gambiae*[7]. According to Chouaibou *et al*[8], changes in mosquito physiology that is not specifically associated with insecticides but that occurs with senescence such as an increase in the rate of cuticle permeability or a decrease in the rate of xenobiotic excretion, could also lead to an increase in susceptibility to insecticides.

The mortality rate obtained when female *An. gambiae* Sekandji populations were unfed and aged 20 days old was higher than the one obtained when these populations were unfed and aged 2-5 days old. This result shows that more the mosquito was old, more he was susceptible to the insecticide. In the current study, a similar pattern was observed when female *An. gambiae* Ladji and Sekandji populations were blood fed and aged 20 days old comparatively to when these populations were blood fed and aged 2-5 days old. A similar pattern was observed in *An. gambiae* Giles from Zanzibar by Lines and Nassor who showed that the mortality rate rose with age when mosquitoes were old[9]. Another similar pattern was also observed in *Anopheles funestus* FUMOZ-R from Southern Mozambique[10]. It has also been observed that older mosquitoes are sometimes less resistant to insecticides, especially when resistance is conferred by the presence of a detoxifying enzyme, the activity of which tends to decline with age[11]. Consequently, it is recommended that susceptibility tests be performed on non-blood fed females, aged no more than 3-5 days post emergence[2]. However, a converse situation was observed in *An. gambiae* Ladji. In fact, the mortality rate obtained when female *An. gambiae* Ladji populations were unfed and aged 20 days old was lower than the one obtained when these populations were unfed and aged 2-5 days old. This result also shows that both *An. gambiae* populations did not tolerate permethrin in the same way regarding their age.

The results obtained after effects of synergist PBO on F1 progeny of *An. gambiae* Ladji populations resistant to permethrin shows that the resistance is a hereditary and dynamic phenomenon which can be due to biochemical mechanisms like overproduction of detoxifying enzymes activity. In fact, the overproduction of mono-oxygenases activity recorded in *An. gambiae* parent mosquitoes from Ladji in 2008 was confirmed in 2013 in these same *Anopheles gambiae* parent mosquitoes from Ladji and in F1 progeny of *An. gambiae* Ladji in 2013[3].

The resistance is a hereditary and dynamic phenomenon which can be due to metabolic mechanisms such as overproduction of detoxifying enzymes activity. Many factors influence vector susceptibility to insecticide. Among these factors, there are mosquito sex, physiological status and mosquito age. Therefore, it is useful to respect the WHO criteria in the assessment of insecticide susceptibility tests in malaria vectors. Otherwise, susceptibility testing is conducted using unfed female mosquitoes aged 3-5 days old. Tests should also be carried out at (25±2) °C and (80±10)% relative humidity.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Comments

Background

Mosquito-borne diseases like malaria affect notable parts of the world. In lack of effective vaccine and some problems due to the drug resistance in pathogens, the vector control plays great role in order to control the mosquito-borne diseases. The application of insecticides is an effective part of vector control programs and determination the susceptibility of the vectors against the insecticides is the first and important step in this process.

Research frontiers

This work describes the effect of the age, physiological status and the sex of the *Anopheles* specimen on the results of the susceptibility tests. Also it tries to explain the possible role of each of these factors in the process of tests.

Related reports

Several reports have been conducted regarding to the susceptibility tests among different mosquito species in different regions, but this work is among the few of them which tries to describe the effect of some complementary mosquito-related factors.

Innovations and breakthroughs

This work focused on the some questionable factors in the process of the mosquitoes susceptibility tests and tries to determine their role.

Applications

The results of this work will help to evaluate the WHO standard process of susceptibility tests and improve it during the time. The determination the role of some factors such as the specimens sex, age and physiological status in susceptibility tests will help the scientists to consider these factors in possible revision of the WHO standard susceptibility tests.

Peer review

This is a good work in which the authors tried to determine the effect of some factors such as sex, age and physiological status of mosquito specimens on the results of the WHO standard insecticide susceptibility tests. The results could be useful for considering these and other probable effective factors during the WHO standard susceptibility tests.

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