INTRODUCTION

Malaria, one of the most devastating human parasitic diseases, is prevalent in tropical developing regions, causing great morbidity and mortality. Despite all control efforts, 3.4 billion people are still at risk of malaria, and approximately 207 million cases of malaria occur globally each year.1 It is well known that human malaria is transmitted exclusively by anopheline mosquitoes. The susceptibility of Anopheles spp. to different human malarial parasites has been widely investigated. In 1929, Hindle and Feng2 studied the artificial infection rate of An. sinensis with the local Plasmodium vivax. Subsequently, more studies were undertaken to observe the development of P. vivax in An. sinensis.3–17 However, other culicine mosquito species, such as Aedes spp, Culex spp, and Mansonia spp, are also abundantly present in malaria-endemic areas. Similar to the anophelines that feed on humans, culicines are important vectors for human disease, transmitting arboviruses and filarial worms. Although culicine mosquitoes have never been shown to transmit human malaria, they can transmit avian Plasmodium species to birds; and several studies have investigated whether different human-infectious Plasmodium spp. can complete their development in culicine mosquitoes. Depending on the mosquito and parasite species used, partial or complete development of Plasmodium has been observed in culicine mosquitoes.18–24 In 1937, Williamson and Zain21 infected Culex tritaeniorhynchus with P. falciparum, P. vivax and P. malariae successfully, representing the first observation of human malarial parasites in a culicine mosquito. Oocysts have been observed in the midgut epithelium in Mansonia uniformis infected with P. falciparum.25
larly, ookinetes have been observed in the blood meals of mosquitoes infected with *Cx. salinarius* and *Cx. quinquefasciatus*. Although, Alavi *et al.* observed *P. berghei* ookinetes in the blood meals of experimental *Ae. aegypti*, the transmission experiment was unsuccessful.

Under certain ecological circumstances, pathogens are able to rapidly adapt to new vectors. For example, the chikungunya virus (CHIKV) was originally transmitted by *Ae. aegypti*. A single point mutation resulting in an amino acid replacement significantly increased the transmission efficiency of the virus in *Ae. albopictus*. *Plasmodium vivax* adaptation to different anophelines in Mexico is also a very good example; in coastal areas, the parasites are transmitted by *An. albimanus*, the most common anopheline in these areas, whereas at higher altitudes, the main vector is *An. pseudopunctipennis*, a foothill mosquito. These examples show that pathogens, including *Plasmodium* spp., are able to adapt to new vectors rapidly following environmental changes.

Due to the great capacity of *Plasmodium* spp. to adapt to new vectors on different continents, ongoing ecological changes caused by humans might promote the adaptation of human-infectious *Plasmodium* parasites to culicines. The transmission of avian *Plasmodium* spp. by anopheline and culicine mosquitoes clearly suggests that such transmission might be possible for mammalian-infectious *Plasmodium* parasites. Based on our current knowledge, it is difficult to predict whether such adaptations will occur, and more research is needed to provide a more reliable prediction.

In China, *An. sinensis* Wiedemann plays a major role in the maintenance of *P. vivax* malaria transmission. *Culex tritaeniorhynchus* Giles is the primary vector of Japanese encephalitis (JE) virus, and *Cx. pipiens pallens* Coquillett is the primary vector of JE virus and filariasis in China. *Anopheles sinensis* Wiedemann, *Cx. pipiens pallens* Coquillett and *Cx. tritaeniorhynchus* Giles (Diptera: Culicidae) are three dominant species in Shandong Province. However, data on the susceptibility of these three mosquitoes to *P. vivax* are rare in Shandong Province, and *Cx. tritaeniorhynchus* and *Cx. pipiens pallens* have never been observed to be infected with *P. vivax* in China. The aim of this study was to investigate the susceptibility and development of *P. vivax* in the three main species of mosquitoes in Shandong Province, China.

**MATERIAL & METHODS**

**Colonization of mosquitoes**

This study was carried out in Jining, Shandong Province, China from July to October 2010 and three mosquito species—*An. sinensis*, *Cx. tritaeniorhynchus* and *Cx. pipiens pallens* were maintained in the laboratory of the Shandong Institute of Parasitic Diseases, Jining. These mosquitoes were reared using the methods described by Park *et al.* in an insectary room at 27 ± 1° C and at 70-80% relative humidity in a 12 h light/12 h dark cycle, and adult mosquitoes were raised with 10% (w/v) glucose with a sponge. For two days prior to blood feeding, the 6- to 8-day-old mosquitoes were provided only water.

**Selection and screening of gametocyte patients**

The only malaria parasite reported in the study area is *Plasmodium vivax*. The patients who sought clinical treatment in Shandong Institute of Parasitic Diseases were screened for malarial parasites by a standard finger prick and Giemsa-stained blood smear. The patients who presented with a high density of *P. vivax* in the blood (48–385 *P. vivax*/100 leucocytes) and with an appropriate gametocyte count (0.5–30/100 *P. vivax*), as observed in a thin blood smear under a microscope, were selected. The screening took place the same day as the experimental infection of mosquitoes. A total of 1.5–2 ml of venous blood from the selected patients was drawn into heparinized tubes for the experimental feedings.

**Experimental infections**

Prior to the infectious blood meal, mosquitoes were starved for 1–2 days in the mosquito cages. Freshly drawn blood was immediately transferred to artificial membrane feeders that had been prewarmed to 37°C, as described previously. Finally, a fresh, washed amniotic membrane was tightly placed on the bowl and allowed to dry in the shade. Before the experiment, the dry amniotic membranes were prepared by moistening them with physiological saline and stretching them across the bottom of a 250 ml wide-mouth bottle, ensuring a proper gap between the amnion and the bottom, and using a rubber stopper with two glass tubes to plug the bottle mouth. During blood feeding, a constant-temperature (37°C) circulating-water system was used to prevent exflagellation of the microgametocytes. Mosquitoes were allowed to feed for 20 min to 2 h each time. After being blood fed, all the engorged mosquitoes were reared for 10 days, and the stomach and the salivary glands of each mosquito were dissected under the microscope and checked for the presence of oocysts and sporozoites.

**Temperature and humidity indoors**

The temperature and humidity were measured and recorded twice daily (800 and 1700 hrs) in the insectary...
during the study period. The average temperature was 16.8–30°C, and the relative humidity was 70–98%. From blood feed to dissection, the average temperature was 20.3–28.8°C in the insectary for 15 batches of mosquitoes.

**Ethical approval**

All human-subject research conducted in this study was reviewed and approved by the Institutional Ethics Committee of the Shandong Institute of Parasitic Diseases (SIPD), Shandong Academy of Medical Sciences. All the patients involved in this study read and signed informed consent forms.

**RESULTS**

**Patient data**

More than 400 symptomatic malaria patients visited the Shandong Institute of Parasitic Diseases in Shandong during the study period. After screening, 185 volunteers were enrolled for the present study.

**Percentage of positive mosquitoes**

For *An. sinensis*, 195 mosquitoes were dissected among which 154 showed ovarian development and 41 were undeveloped (Tables 1 and 2), whereas 26 (13.3%) mosquitoes were positive for *P. vivax*; 11 (5.6%) were positive for oocysts; and 18 (9.2%) were positive for sporozoites. In the 154 mosquitoes with developed ovaries, 10 (6.5%) were positive for stomach oocysts, 18 (11.6%) were positive for salivary gland sporozoites, three were positive for oocytes in the stomach and sporozoites in the salivary glands, and a total of 25 (16.2%) mosquitoes were positive for stomach oocysts and salivary gland sporozoites. Only 1 of 41 (2.4%) of the ovarian-immature *An. sinensis* mosquitoes had an oocyst in the stomach, but sporozoites were not found in the salivary glands.

For *Cx. tritaeniorhynchus*, 203 mosquitoes were dissected (149 showed ovarian development, 54 were undeveloped), and 19 *Cx. pipiens pallens* were dissected (15 showed ovarian development, four were undeveloped). The oocytes and sporozoites were not found in the stomachs or salivary glands in either of these two species of mosquitoes.

In the 11th batch, one blood-fed *Cx. bitaeniorhyncus* mosquito with an undeveloped ovary, and no oocytes or sporozoites were detected. *Cx. bitaeniorhyncus*, however, is not a predominant mosquito species and was not included in Tables 1 and 2.

**Infection condition**

(i) Of the 15 batches of infectious blood that were
fed to the *An. sinensis*, *Cx. tritaeniorhynchus*, *Cx. pipiens pallens* and *Cx. bitaeniorhynchus* species of mosquitoes, only the 7th, 13th, and 15th batches yielded positive results. In the three batches, 125 *An. sinensis* (103 with ovarian development, 22 undeveloped), 60 *Cx. tritaeniorhynchus* (21 with ovarian development, 39 undeveloped); and five *Cx. pipiens pallens* (2 with ovarian development, three undeveloped) were fed to engorgement. The positive rates of infection with *P. vivax* in *An. sinensis* mosquitoes were 21.2, 13 and 36.3% in the three batches of mosquitoes, respectively, with an average of 23.5%; (ii) For the three batches of blood that yielded positive mosquitoes, the experimental blood was taken from patients who experienced malarial attacks 4–5 times per day. Among the three blood batches, two had >15 gametocytes per 100 leukocytes and the remaining batch had six gametocytes. The gametocyte rates were 19, 4 and 3.9% for the 7th, 13th and 15th batches, respectively; and (iii) The days and temperature required for the appearance of sporozoites in the mosquito salivary gland were nine days at an average temperature of 28.2°C in mid- to late August, 12 days at an average temperature of 24°C in mid- to late September, and 17 days at an average temperature of 20.5°C in late September to early October. In this experimental study, the sporozoites and oocysts were observed for last time on the 11 October 2010 (Tables 1 and 2).

### DISCUSSION

From mid-July to early October 2010, individuals from the three predominant species of mosquitoes in Shandong Province, China, *i.e.* 195 *An. sinensis*, 203 *Cx. tritaeniorhynchus* and 19 *Cx. pipiens pallens*, were subjected to artificial infection with the human malarial parasite *P. vivax* via membrane feedings. For *Cx. tritaeniorhynchus* and *Cx. pipiens pallens* mosquitoes, this is the first study to evaluate the potential for infection with *P. vivax* in China.

Total 15 batches of 417 mosquitoes were dissected in three months during 2010. Of these, 26 mosquitoes from three batches totaling 190 mosquitoes (125 *An. sinensis*, 60 *Cx. tritaeniorhynchus* and 5 *Cx. pipiens pallens*) were vivax positive. The results of susceptibility of *An. sinensis* to *P. vivax* were also consistent with earlier experiments2–4, 7, 9. The results proved that the susceptibility of *An. sinensis* to *P. vivax* in Shandong Province is still very high.

The three batches of blood that produced *P. vivax*-positive mosquitoes were obtained from patients who had been attacked by clinical malaria 4–5 times per day, with two batches having >15 gametocytes/100 leukocytes and one batch with 6.4 gametocytes/100 leukocytes. The patient who donated blood for the 15th batch was treated with an eight-day regimen of chloroquine and primaquine twice daily. This finding suggests that, regular treatment of patients infected with malaria parasite is very important to control the spread and prevalence of malaria effectively.

The mosquitoes with complete ovary development were engorged fully. Those that did not fully engorge, failed to do so because the temperature was too low, which affected body fat accumulation, or because the ovaries were not fully developed. For the 15th batch (blood feeding studied in late September, dissection in early October), mosquitoes with undeveloped ovaries accounted for more than half of the number of blood-fed mosquitoes due to the lower room temperature (average 20.3°C).

Although, *P. falciparum* and other human-infectious *Plasmodium* spp. come closest to completing their developmental cycle in culicine mosquitoes18, oocysts, as well as sporozoites, have been also reported in *Mansonina uniformis*19. In infections of *Ma. uniformis* with *P.
falciparum, only oocysts were found in the midgut epithelium25. The ookinetes have been observed in the blood meal of mosquitoes in studies with Cx. salinarus23, Cx. quinquefasciatus26 and Ae. aegypti24. The ookinetes number of P. falciparum in the blood meals of Cx. quinquefasciatus were nearly the same as in their natural vector, An. gambiae20. It remains unclear, however, whether the results were comparable because of the use of different genotypes for the parasite and the vector40–42.

In this study, the locally predominant Cx. tritaeniorhynchus and Cx. pipiens pallens were not infected with P. vivax. The results are consistent with an earlier study on Cx. bitaeniorhynucus and P. falciparum, in which no oocysts were observed in the midgut epithelium43. The transmission experiment with P. berghei in Ae. aegypti was also unsuccessful25. In infections of Ma. uniformis with P. falciparum, no mosquitoes were maintained long enough to determine whether sporozoites would develop in the oocysts and invade the salivary glands25. It is still difficult to determine whether P. vivax infects Cx. tritaeniorhynchus and Cx. pipiens pallens in nature. In nature, avian plasmodia develop in and are typically transmitted by culicine mosquitoes—Culex and Aedes. However, some avian malarial parasites, for example, P. gallinaceum, have been observed of being transmitted by Anopheles quadrimaculatus44–46, An. stephensi, and An. gambiae24, 47–48 under laboratory conditions. In a study also, Plasmodium-refractory and a Plasmodium-susceptible line of An. gambiae were genetically selected, conferring either refractoriness or susceptibility to P. cynomolgi and P. gallinaceum and to some P. falciparum (human malaria parasite) lines47. Similarly, a highly Plasmodium-susceptible line of An. stephensi was selected, and within a few generations, this line showed over 85% infection prevalence in P. gallinaceum48. Another species of avian malarial parasite, P. relictum, has been successfully transmitted between birds by An. quadrimaculatus, An. albimanus46, 49, An. crucians46, and An. freeborni46, 50.

These results demonstrate that the establishment of the parasite in new regions almost always depends on vectorswitches31–52. Nevertheless, human-infectious Plasmodium spp. have only adapted so far to different anopheline species and not to culicine mosquitoes. The ability of mammalian-infectious Plasmodium parasites to develop in culicine mosquitoes is highly dependent on the combination of the mosquito and parasite species used30.

CONCLUSION

The susceptibility of An. sinensis in Shandong Province to P. vivax appeared very high when evaluated with membrane feeding assay under laboratory conditions. Oocysts and sporozoites were detected only in three batches of mosquitoes for An. sinensis infection, out of the 15 batches that were fed infected blood. In these three batches, the positive rate of An. sinensis infection was 21.2, 13 and 36.3%, with an average infection rate of 23.5%. The other two predominant species of mosquitoes, Cx. tritaeniorhynchus and Cx. pipiens pallens, failed to show susceptibility to P. vivax.

Conflict of interest

The authors declare that they have no any conflict of interest.

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