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VARIATION IN VECTOR COMPETENCE FOR DENGUE 2 VIRUS AMONG 24 COLLECTIONS OF *AEDES AEGYPTI* FROM MEXICO AND THE UNITED STATES

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Abstract. *Aedes aegypti* from 24 collections in Mexico and the United States were challenged orally with dengue 2 virus JAM1409 (DEN-2 JAM1409). The vector competence (VC) of the populations ranged from 24% to 83%. Mosquito populations from the Yucatan exhibited greater VC than those from other areas of Mexico. The presence or absence of a midgut infection barrier (MIB) and a midgut escape barrier (MEB) was determined for mosquitoes in each population. The percentage of mosquitoes exhibiting an MIB ranged from 14% to 59%, and those exhibiting an MEB ranged from 4% to 43% in the collections. The MIB and MEB were not completely independent as determined by regression analysis. Midgut infection rates were dose dependent.

INTRODUCTION

Dengue is a resurging disease with approximately 50–100 million cases of classic dengue fever (DF) and 500,000 cases of the more severe dengue hemorrhagic fever (DHF) occurring annually.[1,2] Between 1981 and 1995, there was a four-fold increase in the incidence of DF compared with the total incidence in the preceding 30 years.[2] Dengue is normally thought of as a tropical disease, but in recent years, it has been diagnosed in temperate regions such as the southern United States and northern Mexico.[3]

Dengue viruses (genus *Flavivirus*, family Flaviviridae) have a single-stranded positive sense RNA genome.[4] There are four antigenically distinct dengue virus serotypes. Infection with one serotype confers life-long immunity to infection with the homologous, but not heterologous serotypes. Dengue hemorrhagic fever occurs most commonly in patients with a secondary infection with a dengue serotype heterologous to the primary infection, suggesting a potential for immune enhancement.[5,6] However, DHF has been documented in patients experiencing a primary infection, suggesting that certain viruses may be more virulent and capable of causing DHF without immune enhancement.[2,7] Host factors such as age, immune status, and sex may also be contributing to disease outcome.[8]

The primary vector of dengue is *Aedes aegypti*, a synanthropic, container-breeding mosquito that has adapted well to cohabitation with humans. *Aedes aegypti* has a global distribution in tropical and subtropical areas. Because of its public health importance in vectoring dengue and yellow fever flaviviruses, *Ae. aegypti* has been the subject of numerous vector competence and population genetic studies.[9–20]

Vector competence (VC) is defined as the intrinsic permissiveness of a vector to infection, replication, and transmission of a virus.[21] The VC for arboviruses is associated with a number of anatomic barriers to productive vector infection. These include a midgut infection barrier (MIB), a midgut escape barrier (MEB), and a salivary gland barrier.[22] In potential vectors with an MIB, virus cannot infect and/or replicate in the mosquito midgut cells. This may be due to a lack of cell surface receptors for the virus or to midgut cells being nonpermissive for infection with the virus. Potential vectors with an MEB may allow virus replication in the midgut, even to high titers, but virus is then unable to exit the midgut to cause a disseminated infection. Barriers to infection can vary widely among *Ae. aegypti* populations causing VC for dengue viruses to be variable as well. This, in part, may determine the epidemiology of dengue viruses.

Aedes aegypti populations exhibit considerable genetic variability in VC for flaviviruses,[9–11,13] including dengue 2 viruses.[11,12,14,17,20] The VC for flaviviruses in *Ae. aegypti* is thought to be controlled by at least two genes or sets of genes, one controlling the MIB and the other controlling the MEB.[15,17,20]

Many studies have focused on determining genetic differences in mosquitoes from a wide geographic distribution. More recently, significant genetic variation in *Ae. aegypti* on a smaller scale has been demonstrated in Puerto Rico[16] and in Mexico.[19] In this study, we address potential variation in VC on a regional geographic scale in Mexico. The ultimate goal of this research is to determine if genetic variability in *Ae. aegypti* populations conditions the incidence and severity of DF and DHF outbreaks. If mosquito populations that condition more severe disease are found, there is potential for developing genetic biomarkers for populations that pose undue risk for severe dengue disease. This would allow dengue control programs to focus limited control resources on areas at the greatest risk for severe disease.

MATERIALS AND METHODS

Mosquito collections. Mosquitoes were collected as eggs from sites in Mexico and the United States (Figure 1). Table 1 shows collection sites, mosquito generations challenged orally with dengue-2 virus JAM1409 (DEN-2 JAM1409), the number of repetitions, and the sample size range and mean for each collection. Field collected eggs were hatched to begin colonies. Mosquitoes were raised at a constant temperature of 27°C and 80% relative humidity in an insectary with a 12-hour photoperiod. All experiments were performed on mosquitoes of less than three generations in the laboratory in an attempt to avoid effects of colonization and inbreeding on vector competence.[23] A laboratory colony of *Ae. aegypti* from Puerto Rico provided a highly susceptible mosquito control,[17] which was used to monitor for consistency in the oral challenges.

FIGURE 1. Collection sites of *Aedes aegypti* used in this study.

Virus. The DEN-2 JAM1409 virus, which was isolated in 1983 in Jamaica,[24] and had subsequently been passaged in C6/36 cells, was used in the studies. To prepare stock virus, DEN-2 JAM1409 was amplified in C6/36 cells. A 150-cm^2 flask of confluent C6/36 cells was infected at a multiplicity of infection (MOI) of 1.5 virus particles per cell. Cells were incubated for 14 days at 28°C in L15 medium supplemented with 2% heat-inactivated fetal bovine serum, penicillin (1%), streptomycin (1%), and L-glutamine (1%). Virus was harvested and placed in 0.5-mL aliquots, which were stored at −70°C. For oral challenges, 0.5-mL aliquots were used to infect 75-cm^2 flasks of confluent C6/36 cells at an MOI of 1.5. Virus was incubated for 14 days at 28°C in L15 medium as above, and the medium was changed on day 7. Virus and cells were harvested by scraping with a cell scraper, and medium, virus, and cells were collected in a 50-mL conical centrifuge tube. The virus suspension was mixed 1:1 with defibrinated sheep blood and placed in membrane feeders covered with mouse skin or parafilm membranes.[25] Pre- and post-blood meal virus titers were determined by inoculating serial 10-fold dilutions of the respective meal onto C6/36 cells in the wells of a 96-well plate. Cells were assayed by immunofluorescence, and tissue culture infectious dose 50 ($TCID_{50}$) titers were calculated using the Karber formula.[26] Undiluted virus titers ranged from $10^{7.5}$ to $10^{8.5}$ $TCID_{50}$/mL in all oral challenges. Titers ranged from $10^{5.8}$ to $10^{7.7}$ $TCID_{50}$/mL in the dose-response studies.

Oral challenges. For mosquito infection studies, 100–150 mosquitoes/one-pint carton were starved of sucrose and deprived of water for 24 hours prior to blood feeding. Blood meals were maintained at a constant temperature of 37°C. Mosquitoes were allowed 45 minutes to 1 hour to feed. Fully engorged mosquitoes were selected and held for 14 days at a constant temperature of 27°C and a relative humidity of 80% in an insectary with a 12-hour photoperiod.

Studies were conducted to determine the effect of viral dose on mosquito VC for certain collections. Sequential 10-fold dilutions of virus were used to prepare infectious blood meals: 10^{-0}, 10^{-1}, and 10^{-2}. Fully engorged mosquitoes were selected and held for 14 days at a constant temperature of 27°C and a relative humidity of 80% in an insectary with a 12-hour photoperiod. Pre-blood meal and post-blood meal titers for each of the dilutions were determined as previously described.[26]

Vector competence. Mosquitoes were phenotyped for VC using immunofluorescence at 14 days post oral challenge. Mosquito heads were severed, squashed onto acid-washed slides, fixed in acetone, and assayed for dengue virus by indirect immunofluorescence. Slides were incubated for 40 minutes at 37°C with a mouse-derived primary monoclonal antibody directed against a flavivirus E gene epitope[27] diluted 1/200 in phosphate-buffered saline (PBS). Slides were then washed twice in PBS. A mixture of biotinylated sheep anti-mouse antibody (Amersham, Arlington Heights, IL) and Evans blue (1%) as a counterstain was made at a dilution of 1/200 in PBS. Slides were incubated for 40 minutes at 37°C followed by two washes in PBS. Slides were then incubated 10 minutes with streptavidin fluorescein (Amersham) diluted 1/200 in PBS to detect the primary-secondary antibody complexes. Slides were washed twice in PBS and once in water. Glycerin containing diazobicyclo(2,2,2)octane (DABCO D-2522; Sigma, St. Louis, MO) was applied, and a coverslip

TABLE 1
Aedes aegypti collections assayed for vector competence (VC)*

Country/State of collection	City of collection	Generations used	No. of heads infected/no. tested	No. of repetitions	Sample size range	Mean sample size
Puerto Rico	Laboratory colony	Unknown	460/568	12	18–63	47
Mexico						
Sonora	Hermosillo	F1	25/55	2	14–41	28
	Guaymas	F1	24/39	2	17–22	20
Sinaloa	Culiacan	F1, F2, F3	119/151	4	15–60	38
	Mazatlan	F1	54/84	5	10–25	17
Jalisco	Puerto Vallarta	F1	24/80	2	18–62	40
Colima	Manzanillo	F1	66/117	3	26–63	39
Michoacan	Lazaro Cardenas	F1	48/105	2	43–62	53
Guerrero	Ixtapa Zihuatanejo	F1	16/68	2	25–43	34
	Coyuca de Benitez	F1	33/45	3	10–19	15
Oaxaca	Puerto Escondido	F1, F2	89/148	4	15–63	37
Chiapas	Tapachula I	F1, F2	78/108	2	47–61	54
	Tapachula II	F2, F3	50/100	4	12–37	25
Quintana Roo	Chetumal	F1, F2	90/109	2	49–60	55
	Cancun	F1, F2	100/139	3	19–60	46
Yucatan	Merida	F1, F2	101/149	3	44–60	50
Campeche	Campeche	F1	26/60	1	60	60
	Ciudad del Carmen	F1	26/60	1	60	60
Tabasco	Villahermosa	F1, F2, F3	85/175	4	17–60	44
Veracruz	Moloacan	F1, F2	70/123	3	20–60	41
Tamaulipas	Miguel Aleman	F1, F2	117/196	4	20–60	49
	Nuevo Laredo	F1, F2	82/171	3	48–63	57
Nuevo Leon	Monterrey	F1, F2	69/121	3	19–60	40
United States/Texas	Houston	F1, F2	39/100	2	40–60	50
Arizona	Tucson	F1, F2	64/99	2	39–60	50

* Shown are mosquito strain designation, collection site, colony generations challenged, number of infected heads per total number tested (VC), number of repetitions, the sample size range, and the mean sample size for each repetition. A total of at least 60 mosquitoes were assayed for phenotype in all mosquito collections except Guaymas, Hermosillo, and Coyuca de Benitez. All collections except Ciudad del Carmen and Campeche were challenged more than once. All mosquitoes challenged were less than three generations in the laboratory.

placed on each slide. Heads were examined at ×200 using an Olympus (Melville, NY) BH2-RFCA microscope. If virus antigen was not detected in head tissues, the respective abdomens, which were stored at −70°C, were subsequently assayed by immunofluorescence for virus antigen.

Detection of virus antigen in midgut tissue revealed a midgut infection (MI) and the midgut infection rate (MIR) was calculated as the number of positive midguts divided by the number of mosquitoes exposed. Detection of virus antigen in head tissue revealed a disseminated infection (DI), and the disseminated infection rate (DIR) was calculated as the number of mosquitoes with virus antigen in the head tissue divided by the number of mosquitoes with virus antigen in the midgut. The VC was calculated as the number of mosquitoes with DI divided by the number of mosquitoes exposed. Mosquitoes were not assayed for salivary gland infection or for actual virus transmission; the VC was assumed to be the same as the rate of virus dissemination to the head tissues. Once mosquitoes were phenotyped for MI and DI, the percent mosquitoes exhibiting a MIB was calculated as 1 - MIR. The percentage of mosquitoes with an MEB was calculated as the MIR - DIR.

RESULTS

Variation in VC. The VC of mosquitoes from the 24 geographic collections sampled are shown in Figure 2. These populations were previously geographically grouped as Pacific, Yucatan, or Northeast.[28] Error bars indicate the standard error among replicates of oral challenges for the respective collection. All results are based on a sample size of greater than 60 mosquitoes per collection, with the exception of collections from Guaymas, Hermosillo, and Coyuca de Benitez, and most are based on composite results of more than one oral challenge (Table 1). Oral infection rates were consistent between challenges. The VC for the Puerto Rico strain ranged from 72% to 96%, and did not differ significantly by chi-square analyses ($P < 0.05$). The VC ranged from 24% in the Ixtapa Zihuatenejo collection to 83% in the Chetumal collection. Interestingly, the mosquito populations from the Yucatan exhibited a greater overall VC (62%) than mosquito populations from the Northeast (53%) and the Pacific (57%) (Table 1; $P < 0.005$). The VC of the Yucatan populations appeared less variable than that of the other geographic collections. The Yucatan collections clustered into two groups, which differed significantly in VC by chi-square analysis ($P < 0.001$); 73% (291 of 397) of the mosquitoes from Quintana Roo and Yucatan states and 46% (137 of 295) of the mosquitoes from Campeche and Tabasco states were capable of transmitting virus.

The VC was more variable in the Northeastern and Pacific collections, and significant differences in VC were detected in geographically proximal collections. For example, the VC of mosquitoes from Coyuca de Benitez was 73%, while the VC of mosquitoes from Ixtapa Zihuatanejo was 24% (Table 1). These rates differed significantly by chi-square analysis ($P < 0.001$) despite the fact that the two collections are proximally close and in the same state (Figure 1). Thus, geographic location was not necessarily correlated with VC.

Anatomic basis of transmission potential. To determine anatomic barriers that condition transmission potential, mosquitoes from the 24 experimental *Ae. aegypti* collections and the Puerto Rico (susceptible) control were phenotyped for the presence of MIB and MEB. The collections exhibited a

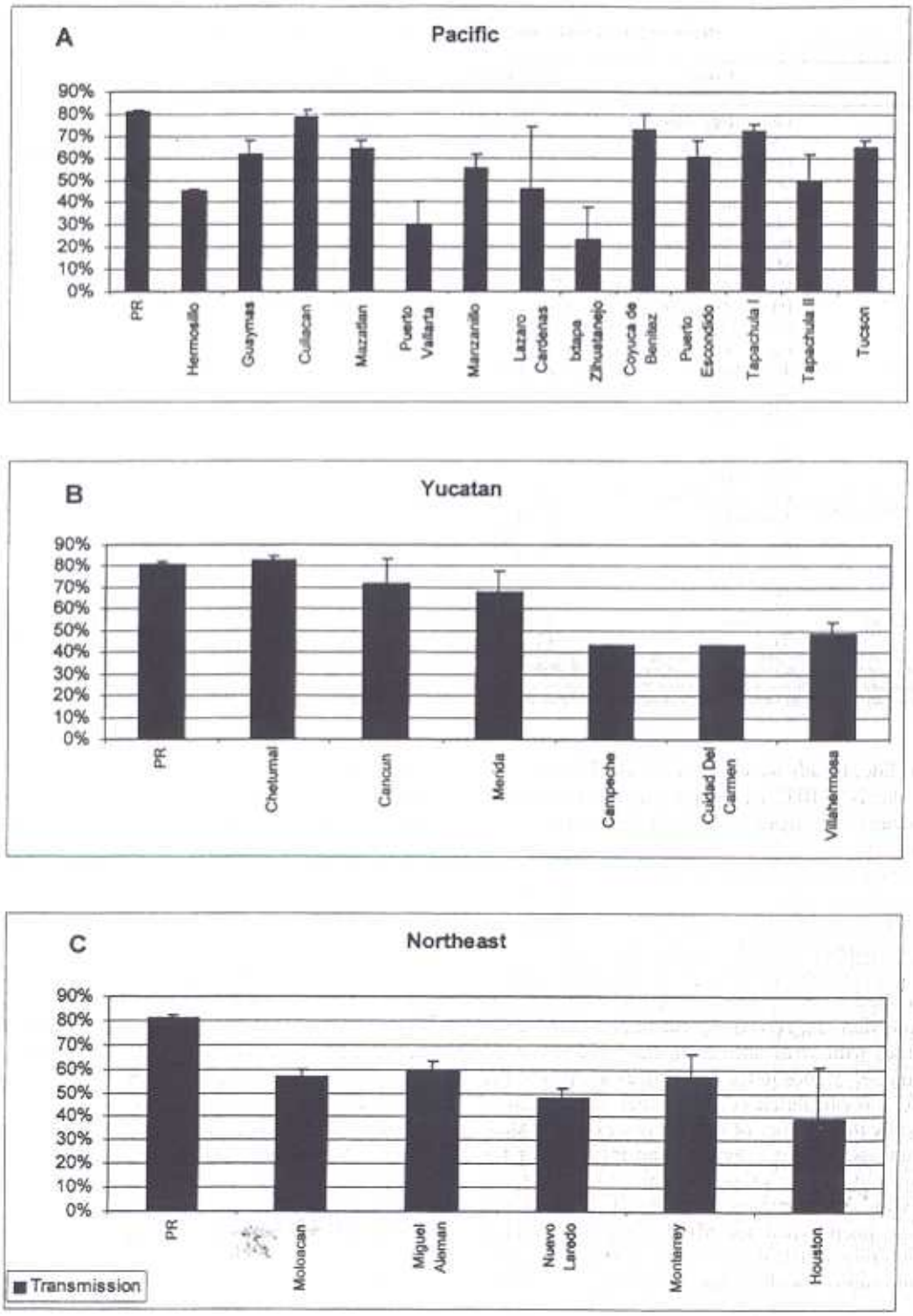

FIGURE 2. Vector competence (VC) of *Aedes aegypti* collections for dengue 2 JAM1409 virus expressed as the percentage of the total number of individuals tested that displayed disseminated infection. Collections are arranged according to geographic areas designated by Gorrochotegui-Escalante and others.[28] These include collections from the Pacific coast (**A**), collections from the Yucatan Peninsula (**B**), and collections from northeastern Mexico (**C**). Error bars indicate standard error of challenge experiments. Where there are no error bars, collections were challenged only once (Campeche and Chetumal) or there was no difference in VC among challenges.

wide range of expression in MIB and MEB for DEN-2 JAM1409 (Figure 3). The Ixtapa Zihuatanejo mosquitoes exhibited the largest MIB (59%) as well as the largest MEB (43%). Mosquitoes from Chetumal had the lowest MIB (14%) and the lowest MEB (4%).

Correlation between MIB and MEB. Figure 4 shows the percentage of mosquitoes exhibiting an MIB versus those exhibiting an MEB. This was performed to determine whether MIB and MEB are completely independent of one another or whether there is a correlation between them. Regression

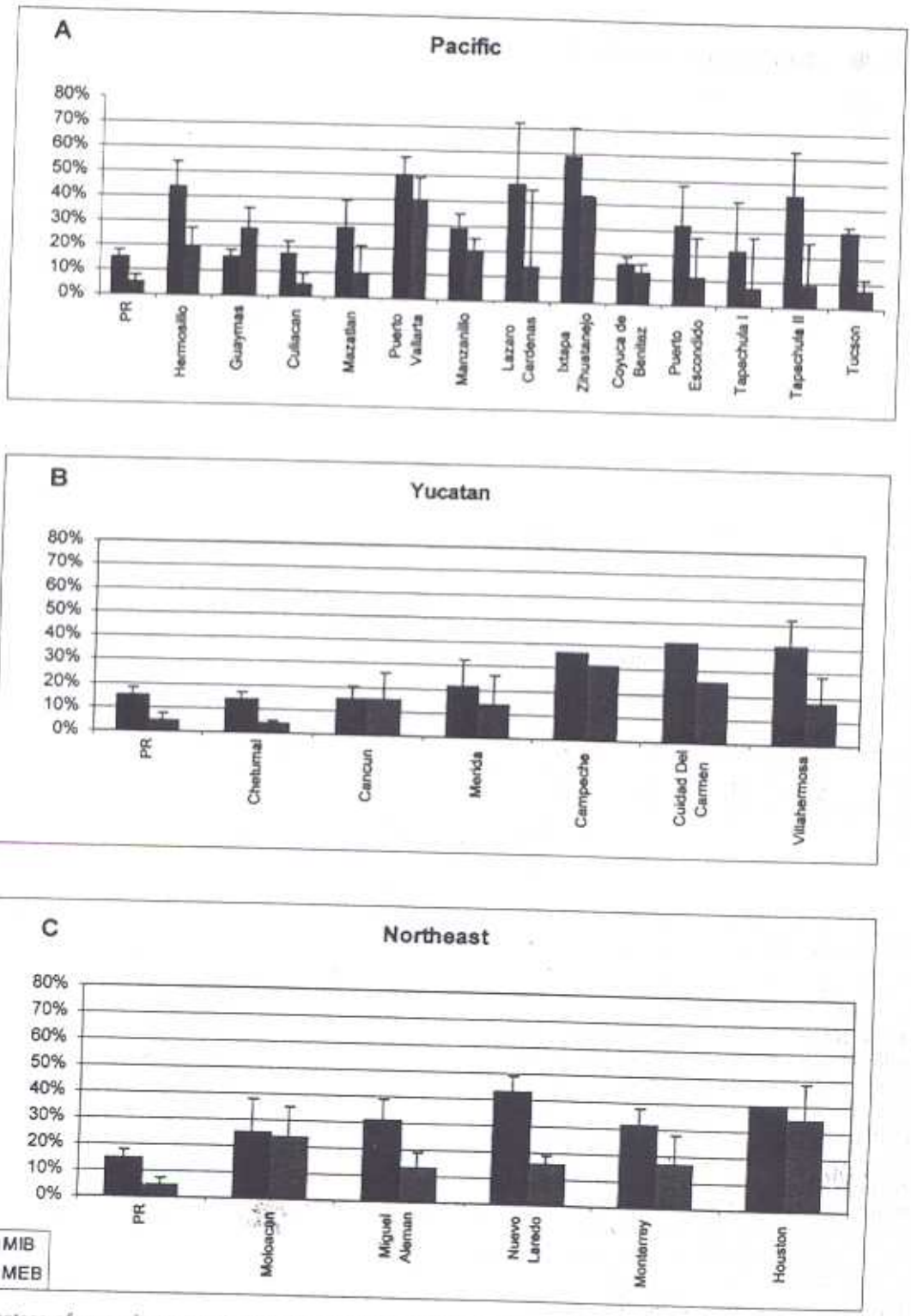

FIGURE 3. Percentage of mosquitoes tested that exhibit a midgut infection barrier (MIB) and a midgut escape barrier (MEB) for each collection. Collections are arranged according to geographic areas designated by Gorrochotegui-Escalante and others.[28] These include collections from the Pacific coast (**A**), collections from the Yucatan Peninsula (**B**), and collections from northeastern Mexico (**C**). Error bars indicate standard error of challenge experiments. Where there are no error bars, collections were challenged only once (Campeche and Chetumal) or there was no difference in VC among challenges.

analysis gives r values for the different geographic groupings that indicate a weak correlation between MIB and MEB. However, this correlation is only significant by Pearson's chi-square goodness of fit test in the Pacific grouping ($P < 0.05$) and for all sites grouped together ($P < 0.01$).

Dose response. The effect of virus dose on MIRs was determined for selected mosquito collections (Figure 5). MIRs were directly correlated with virus dose. In each collection, infection rates increased as virus titer increased. However, the dose response was not consistent among collections; some

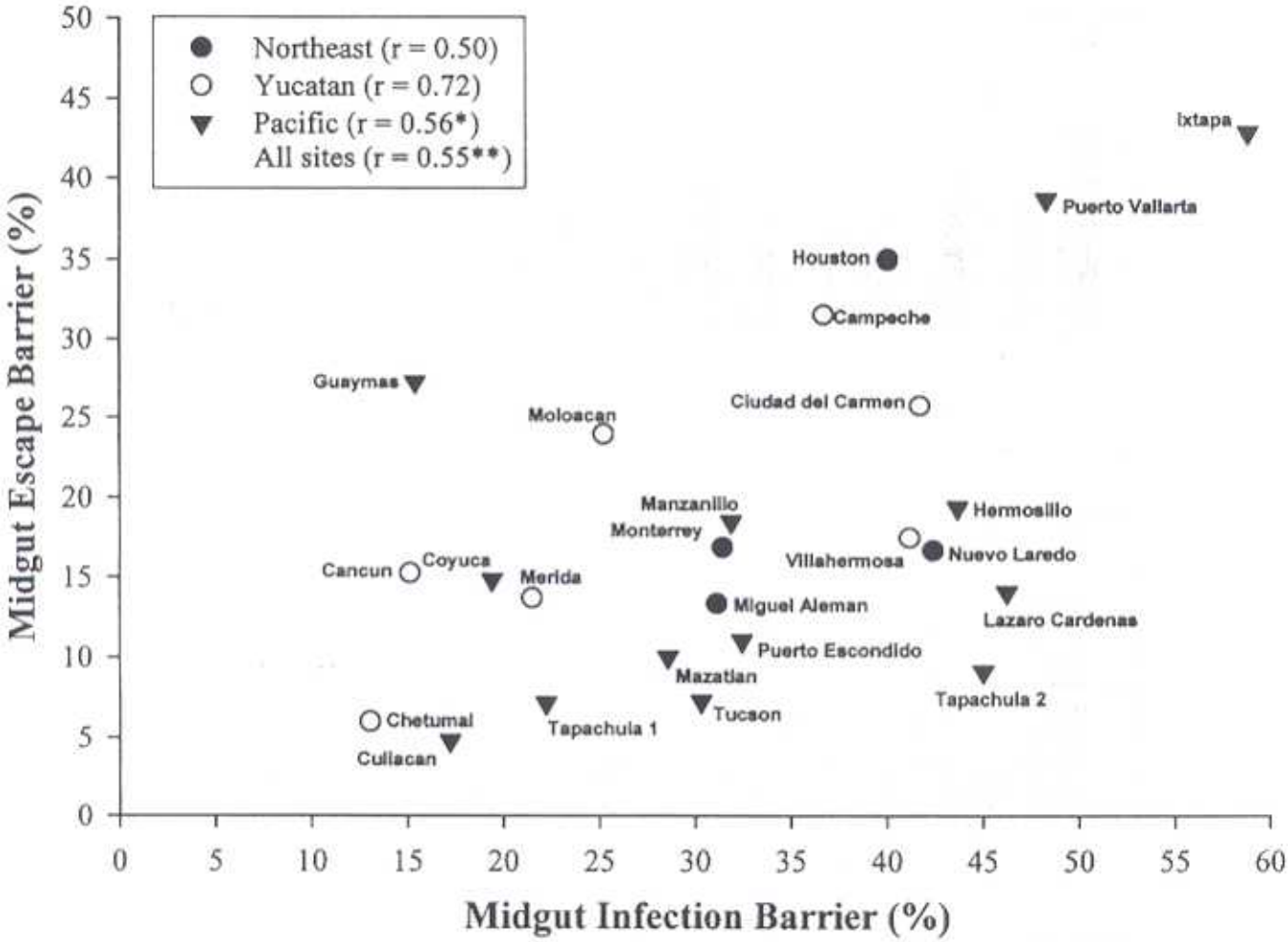

FIGURE 4. Regression analysis of midgut infection barrier (MIB) versus midgut escape barrier (MEB). Collections were plotted based on the percentage of individuals exhibiting an MIB or MEB. Regression analysis was performed for all collections within the designated geographic locations as well as for all collections combined. One asterisk denotes a significance value of $P < 0.05$ and two asterisks denotes a significance value of $P < 0.01$ using Pearson's chi-square goodness of fit test.

infection rates decreased rapidly with lowered virus titer. The Chetumal collection exhibited a gradual decrease in MIRs (90%, 88%, and 61%), as did Tucson (67%, 50%, and 36%). In contrast, the Nuevo Laredo MIRs (60%, 27%, and 14%) and Merida MIRs (57%, 27%, and 14%) rapidly decreased. The DIRs were not correlated with virus dose in infectious feeds. The undiluted virus that was used to challenge mosquito collections from Miguel Aleman and Nuevo Laredo were lower than that used for the other challenges. For this reason, Figure 5 shows infection rates based on the titer of virus in the infectious blood meal rather than based on the dilution factor.

DISCUSSION

In general, the *Ae. aegypti* collections from throughout Mexico exhibited considerable variability in VC, and collections from the Yucatan Peninsula were generally more vector competent than those from other geographic regions (Figure 2). Interestingly, collections from the Eastern Yucatan Peninsula exhibited greater VC than those from the Western Yucatan Peninsula. The anatomic and genetic determinants of these phenotypic differences are being investigated. *Aedes aegypti* collections from throughout Mexico differ significantly in their midgut susceptibility to infection with DEN-2 JAM1409 (Figure 3). Mosquito collections exhibited MIBs that range from 59% in the Ixtapa Zihuatanejo collection to 14% in the Chetumal collection, MEBs that range from 43% in the Ixtapa Zihuatanejo collection to 4% in the Chetumal collection, and VCs that range from 24% in the Ixtapa Zihuatanejo collection to 83% in the Chetumal collection (Figure 2). In general, the Eastern Yucatan Peninsula collections, which exhibited high VC, had low MIBs and generally low MEBs.

Results shown in Figure 3 seem to suggest that VC in the majority of these collections is primarily conditioned by an MIB. This may be due in part to the fact that those mosquitoes with an MIB cannot be tested for the presence of an MEB because there is no midgut infection. Mosquito collections from Chetumal and Culiacan did not have either a strong MIB or a strong MEB. An extremely high VC in both of these collections is evidence for this. Ixtapa Zihuatanejo had a strong MIB and MEB, and its VC was significantly lower than that of Chetumal and Culiacan ($P < 0.001$). Over-

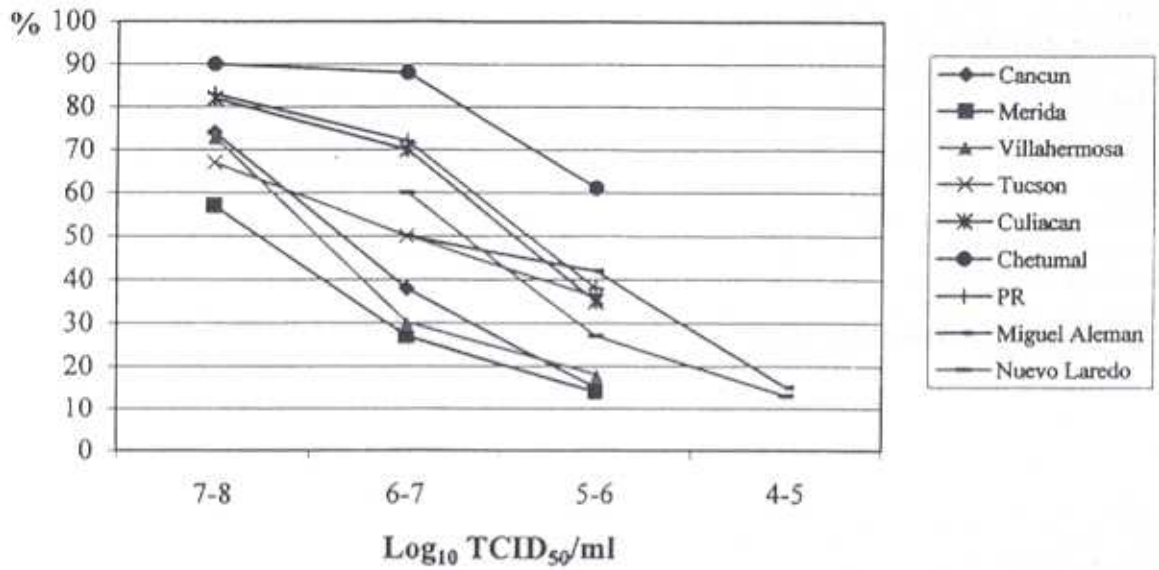

FIGURE 5. Dose dependency of mosquito midgut infection rates with dengue 2 virus. Mosquitoes were exposed to serial 10-fold dilutions of DEN-2 JAM1409 virus in blood meals, and subsequently assayed for midgut infection. The x-axis denotes virus titers in $\log_{10}$ tissue culture infectious dose 50 ($TCID_{50}$) titers/ml.

all, the VC can be thought of as being inversely proportional to the level of MIB and MEB found in the mosquito collection: strong MIBs and MEBs reduce the potential for transmission.

The perceived range of susceptibilities suggests that the ability to overcome the MIB and MEB to transmit DEN-2 JAM1409 is a quantitative trait with multiple genes that likely condition VC and collectively determine the infection rates of mosquito populations. This concept is not new, and has been studied using crosses of susceptible and refractory mosquito lines.[15,17]

It is interesting that the geographic origin of the *Ae. aegypti* collections is not necessarily indicative of infection rates (Figure 2). Certain collections in close proximity do not exhibit similar infection rates. This can be seen in collections from Ixtapa Zihuatanejo and Coyuca de Benitez, which are geographically close but have significantly different MIBs, MEBs, and VC ($P < 0.01$). A detailed genetic analysis is needed to determine if these collections are actually isolated and constitute independent populations that differ in VC. Some areas exhibit similar infection rates. Collections from Villahermosa, Ciudad del Carmen, and Campeche are located near one another and do not differ in MIBs, MEBs, and VC. To determine whether these are distinct populations, an analysis of gene flow similar to that discussed below should be completed on these collections.

Patterns of gene flow in *Ae aegypti* populations could influence infection rates of proximally close populations. Gorrochotegui-Escalante and others recently showed that gene flow among *Ae. aegypti* populations in Mexico varied by region as determined by the mitochondrial gene ND4.[28] They found high rates of gene flow in populations from the Pacific coast. These populations also had a large genetic diversity. Populations from the Yucatan Peninsula had variable genetic diversity. Many of the mosquito collections used were the same as those characterized for VC in this study; however, there does not seem to be an overall correlation between the mosquito phylogenies determined by Gorrochotegui-Escalante and others[28] and the infection rates reported in this paper. Other genes are currently being analyzed to determine potential correlates with VC.

Previous studies have revealed quantitative trait loci (QTLs) that condition differences in VC and MIB and MEB.[17,20] Whether these condition dengue transmission in these natural populations remains to be determined. It is important to remember that there is potential for different loci to affect VC in different populations. That is, QTLs discovered in previous studies based on crosses of laboratory colonies may not be pertinent to field populations, which may have their own QTLs acting on VC for dengue. Thus, the importance of looking for novel QTLs and genetic variation is evident.

The regression analyses (Figure 4) show that the MIB and MEB do have some degree of correlation (r values ranging from 0.50 to 0.72). This correlation may suggest the presence of an overall antiviral response from the mosquito that inhibits both midgut infection and midgut escape. If, for example, a mosquito responded to virus by inducing apoptosis of infected cells, this could potentially affect both midgut infection and escape, and would help explain why the two are correlated. However, the MIB and MEB are not tightly correlated, and this leaves room for independently acting factors to affect either midgut infection or midgut escape. This again stresses the importance of mapping QTLs associated with MIB, MEB, or both.

In each collection, the MIRs were dose dependent (Figure 5). The dose-response curves, which reflect the thresholds of infection, differed slightly for each collection.[22] The DIRs did not correlate with virus dose in infectious blood meals. This is not surprising since Bosio and others previously reported that virus dissemination rates in *Ae. aegypti* do not correlate to midgut virus titer.[17] The dose response results suggest that the high titers used in the VC studies do not obscure differences in susceptibility among the collections. In most cases, the collections retain the same VC ranking through the different virus titers (Figure 5). Where they do change order, the differences are not great.

In future studies, we will determine if different DEN-2 virus genotypes and other DEN serotypes yield the same vector phenotypes as DEN2 JAM1409 virus. This would allow us to determine whether mosquito populations differ in their susceptibility to different DEN virus strains or genotypes. If mosquitoes vary in their ability to transmit different DEN genotypes or serotypes, VC may be a major determinant of which genotypes/serotypes do circulate and thus are more prevalent in a given area. Specific genotypes or serotypes would, in this case, be selected for by the vector.

These studies with DEN2 JAM1409 and *Ae aegypti* mosquito collections lay the groundwork for genetic analysis of mosquito determinants of VC. Identified QTLs that condition VC can be used to perform marker-assisted selection (MAS).[17,20,29,30] This would greatly facilitate studies on the mosquito genetics aspect of virus-vector interactions.

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