

Experimental Evaluation of the Mechanical Transfer of Rotavirus A RNA by Female *Culex molestus* Mosquitoes

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Abstract

Background: Rotavirus A is a major cause of acute gastroenteritis, particularly in children and primarily transmitted via the fecal-oral route, but it can also survive and spread indirectly through contaminated surfaces, materials, and water. Despite widespread interest in the role of insects in the mechanical transmission of enteric infectious agents, regarding the potential contribution of mosquitoes, the information on rotavirus A RNA mechanical transfer remains limited successful detection, and studies on this topic are scarce. **Aim:** This study aimed to evaluated the mechanical transfer of rotavirus RNA by *Culex molestus* mosquito's female and the effect of exposure duration on transfer under controlled laboratory conditions. **Materials and Methods:** The study included 75 fecal samples that underwent initial screening for rotavirus antigen using a rapid immunoassay. Molecular detection of viral RNA was then performed using RT-qPCR targeting NSP3 gene. Mosquitoes Female were divided into independent experimental groups and exposed to positive fecal sources for 1, 4, and 24 hours. mosquitoes were transferred to molecularly negative fecal medium., and the mosquitoes were allowed contact with the medium for 8 hours. Ct values, viral loads, and recovery rates were compared among exposure groups. **Results:** Rapid testing showed positive results in 75 samples, while RT- qPCR confirmed the presence of rotavirus RNA in 41 samples, with a molecular positivity r ate of 54.7%.



Mechanical transfer testing revealed the successful detection of rotavirus RNA in the receiving media after all exposure durations, while all negative controls remained RT-qPCR negative. The mean Ct gradually decreased from 0.41 ± 35.84 after 1 hour to 0.36 ± 31.72 after 4 hours, and then to 0.28 ± 27.48 after 24 hours. Concurrently, the estimated viral load increased from $10^1 \times 1.1$ to $10^2 \times 1.7$ and then to $10^3 \times 3.3$ copies/ μL , respectively. The percentage of molecular recovery also increased from 0.11% after 1 hour to 1.7% after 4 hours, and then to 33.0% after 24 hours. Analysis of variance showed a highly significant effect of exposure duration on Ct values ($P < 0.001$). Correlation analysis showed a strong inverse relationship between exposure duration and Ct values ($r = -0.923$, $P < 0.001$). Similarly, the relationship between baseline and post-transfer Ct values showed a strong positive correlation ($r = 0.94$, $P < 0.001$). **Conclusion:** The current study provides experimental evidence for the ability of female *Culex molestus* mosquitoes. The results also showed that the amount of viral genetic material transferred was related to the duration of mosquito exposure to the contaminated source, with longer exposure resulting in lower Ct values and higher estimated viral load and molecular recovery rates. The findings suggest that the viral load in the original source may be an additional factor influencing recovery efficiency after transfer. However, the results demonstrate the transfer of viral RNA but not the transfer of live, infectious viral particles. These findings should be interpreted as evidence of mechanical transfer of viral RNA rather than evidence of vector competence or biological transmission. This was among the first controlled experimental studies investigating the mechanical transfer of rotavirus A RNA by *Culex molestus* mosquitoes.

Keywords: Rotavirus A; Mechanical transmission; *Culex molestus*; Mosquitoes; RT-qPCR; Viral RNA; Ct value; Duration of exposure; Molecular recovery.

Introduction:

Infectious diarrheal diseases are a significant global health problem. Viral, bacterial, and parasitic agents contribute to a large number of illnesses, hospitalizations, and deaths. The burden varies across countries depending on income level, health coverage, and the availability of safe water, sanitation, and immunization services. Rotavirus remains one of



the leading viral causes of severe acute gastroenteritis in young children [1,2]. Although rotavirus vaccines have been highly successful in reducing severe cases, hospitalizations, and deaths, the virus has not been eradicated and continues to circulate in communities to varying degrees. Clinical, molecular, and environmental surveillance remains essential, particularly in settings with poor sanitation or low immunization coverage. Global burden analyses have shown that the introduction of rotavirus vaccines has significantly contributed to reducing rotavirus-related deaths, but the remaining disease burden is still geographically uneven and is more concentrated in resource-limited areas [2,3].

Rotavirus belongs to the non-enveloped enteroviruses and is characterized by a complex viral structure with a three-layered protein capsid surrounding an 11-segment double-stranded RNA genome [4]. Rotavirus transmission occurs primarily via the fecal-oral route, either through direct contact or indirectly via contaminated hands, surfaces, objects, water, and materials. Therefore, the viral transmission cycle can include a series of interactions between the fecal source and the environment, surfaces, and intermediate materials capable of carrying and transmitting contaminants [4,5]. Being an unenveloped virus with a multilayered capsid, it can withstand a range of environmental conditions, allowing viral material or viral particles to survive in contaminated water, surfaces, and organic matter. Studies of enteroviruses have shown that the pathogen's survival outside the host is affected by temperature, humidity, pH, organic matter composition, and surface characteristics [5,6].

Comparative studies between rapid tests and molecular techniques have shown that RT-qPCR can detect viral RNA in samples where rapid antigen tests are negative, due to differences in analytical sensitivity and the duration of molecular signaling. [7,8]. Advances in molecular diagnostic techniques have improved the detection of rotavirus in clinical and environmental samples. [9,10]. Some insects and arthropods can play a role in the physical transmission of pathogens or their components between sources of contamination and various receptors. This mode is known as mechanical transmission, and it differs from biological transmission in that it does not require replication, development, or dispersal of the pathogen within the vector's body. Mechanical transmission can occur



on the legs, body hairs, mouthparts, or through contaminants attached to the insect's body surface, which are then redeposited at another location as a result of movement or repeated contact [11,12]. Most studies on the mechanical transmission of enteric pathogens have focused on insects that live in commensal environments with humans, such studies have shown that insect-borne contaminant transfer is influenced by the concentration of the pathogen at the source, the nature of the contact, the number of landings and movements, the characteristics of the insect's external surface, environmental conditions, and the time interval between acquisition and deposition [13,14].

For a virus to successfully infect mosquito tissues, it must overcome anatomical and immunological barriers and spread to associated tissues. Therefore, the concept of efficient transmission relies on a complex biological compatibility between the virus and the mosquito, and differs fundamentally from the mere physical transfer of a contaminant from one source to another [15,16]. Among the medically and environmentally important mosquito species, *Culex molestus* is a widespread species characterized by significant ecological and behavioral flexibility. *Culex molestus* is well-adapted to environments associated with human activity, including some enclosed and semi-enclosed urban habitats and underground sites. Recent studies have shown that this species is highly adaptable to urban environments, reflecting distinctive environmental and behavioral characteristics that contribute to its persistence and spread in these habitats [17,18]. The environmental characteristics of this mosquito make it scientifically feasible to test its potential contribution to the mechanical transport of viral material upon direct exposure to contaminated sources [12,14]. Global surveillance studies have shown that rotavirus remains of epidemiological importance despite advances in vaccines, making the understanding of potential environmental pathways for viral or genome movement a worthwhile area of research, especially in environments where pollution sources and insect density overlap [3,8]. While there is a wealth of research on rotavirus epidemiology, conventional transmission methods, environmental stability, and molecular diagnostics, information on the mechanical transmission of rotavirus A RNA by mosquitoes, particularly *Culex molestus*, remains very limited compared to the accumulated knowledge on the biological transmission of viruses by mosquitoes and the mechanical transmission



of enteric pathogens by flies. This gap reveals the need for controlled experimental studies that distinguish between the mere presence of viral material in the environment and its actual transfer from a known source to a known recipient via a specific insect medium.

Experimentally, studying the effect of exposure duration to the contaminating source can provide a deeper understanding of the dynamics of acquisition and transfer. However, a distinction should be made between exposure duration and viral retention time; the former measures the effect of the length of contact with the contaminating source, while the latter requires an independent design to establish how long the viral material remains on the insect after the source is removed. Accordingly, the current study aimed to evaluate the mechanical transmissibility of rotavirus A genetic material by female *Culex molestus* mosquitoes under controlled laboratory conditions, and to study the effect of the duration of insect exposure to the positive fecal source on the amount of viral RNA transferred to a previously negative medium, using RT-qPCR. The study hypothesis is based on the assumption that increasing the duration of mosquito contact with the contaminated source will lead to an increase in the amount of viral RNA recovered in the receiving medium, which will be reflected in a decrease in Ct values and an increase in the estimated viral load, while the negative controls remain free of amplification.

Materials and Methods

1 -Study Design

The design of laboratory experimental study consisted of two interrelated phases. The **first phase** involved screening for rotavirus A in 75 fecal samples using a rapid antigen test, followed by molecular analysis using RT-qPCR. The current field study involved collecting (75) stool samples from patients (both outpatients and inpatients) admitted to Bint Al-Huda Teaching Hospital in Dhi Qar Governorate between January 28, 2025, and April 28, 2025. The samples were collected from patients of all ages and both sexes who presented with clinical symptoms consistent with viral gastroenteritis, such as diarrhea, vomiting, fever, abdominal pain, or symptoms consistent with enterovirus infection, as assessed by the attending physician. Approximately 2-3 grams of stool were collected for



each sample to ensure a sufficient quantity of viral particles. It is preferable to examine the stool sample within the first few hours of collection, and to temporarily store it at 2–8°C if it has not been examined directly. Long-term storage is done by freezing at –20°C for molecular examinations. The **second phase** involved using molecularly positive fecal samples with high viral loads as sources of viral contamination in the mechanical transfer experiment. The study indicates that 75 fecal samples were selected for the rotavirus pathway and subjected to molecular detection of viral RNA. In the experimental phase, independent groups of female mosquitoes were exposed to the positive fecal source for 1 hour, 4 hours, or 24 hours, then the positive source was replaced with a negative fecal medium, and the mosquitoes were allowed to come into contact with it for a fixed period of 8 hours. The receiving medium was then examined using RT-qPCR to detect rotavirus RNA transmission.

2-Ethical and Biosafety Considerations

The study was conducted after obtaining the necessary ethical and institutional approvals, and informed consent was obtained from participants or their guardians before sample collection. Identification codes were used instead of personal names when processing data and samples. Stool samples were treated as potentially infectious human materials, and handling was carried out according to Biosafety Level 2 precautions (BSL-2), including the use of gloves, lab coats, and masks, and the disposal of biological waste according to established safety procedures.

3- Inclusion and Exclusion Criteria

Inclusion Criteria: Severe or watery diarrhea, Vomiting or fever accompanying the diarrhea., Clinical suspicion of a Rotavirus A infection., Consent from the patient or their guardian to participate and Availability of stool samples for rotavirus diagnosis, suitable for rapid testing or preservation for molecular analysis.

Exclusion Criteria: Insufficient sample size, Stool samples mixed with urine or externally contaminated, Samples preserved with preservatives such as formalin, Unlabeled samples



or those with incomplete data, Patients who received treatment that may affect the interpretation of results, if this is documented on the clinical information form and Stool Sample Collection for Rotavirus

4 -Rapid Test for Rotavirus in Stool Samples

The Rotavirus Antigen Rapid Test Cassette (Spinreact- Spain)/Colloidal Gold is used for the qualitative detection of rotavirus antigen in stool samples.



Images (1, 2) illustrate the rapid rotavirus test

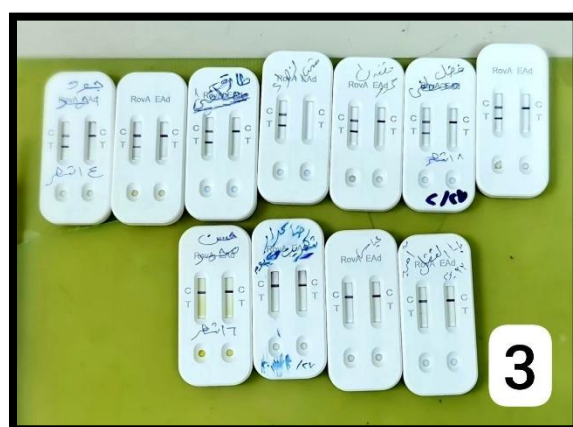


Image (3) shows some results of the rapid rotavirus test



5- Extraction of Viral RNA: The samples were then centrifuged to remove coarse particles, and the filtrate or supernatant was used to extract RNA. The innuPREP Virus DNA/RNA Kit – IPC16, (Innuscreen / Analytik Jena Germany), was used. This kit is designed to isolate viral DNA and RNA from various samples, including serum, plasma, cerebrospinal fluid, stool, and swabs. It includes protocols for extracting viral RNA from 200 μ L of stool or cell-free fluid samples.

6- RT-qPCR Test for Rotavirus Detection

This study utilized the NSP3 TaqMan RT-qPCR Assay (Fisher Scientific / Applied Biosystems United States) to detect viral RNA in stool samples. The RT-qPCR reaction was prepared with a final volume of 20 μ L per sample as follows:

Component	Recommended Volume per Reaction
2 $\times$ One-step RT-qPCR Probe Master Mix	10 μ L
Forward primer	Final concentration: 400 nM
Reverse primer	Final concentration: 400 nM
Probe	Final concentration: 200 nM
Enzyme mix / RT mix	According to the manufacturer's instructions
RNA template	5 μ L
Nuclease-free water	Add to a final volume of 20 μ L

Thermal Amplification Program:

Step	Temperature	Time	Number of Cycles
Reverse transcription	50°C	30 min	1
Initial denaturation / enzyme activation	95°C	10 min	1
Denaturation	95°C	15 sec	40–45
Annealing/Extension + fluorescence acquisition	56–60°C	60 sec	40–45

Quality control Criteria for RT-qPCR Results

RT-qPCR reaction results were accepted according to the reaction's quality control criteria. A sample was considered positive when a normal amplification curve appeared



and a Ct value ≤ 38 was recorded for both viruses, with the positive and negative controls successfully passing the test. Samples that did not show an amplification curve or recorded a Ct value > 38 were considered negative. In cases where borderline Ct values or an atypical amplification curve appeared, the sample was retested to confirm the result. For runtime results to be accepted, the quality controls had to achieve the expected results, including:

1. The positive control successfully yielded clear amplification.
2. No amplification appeared with the negative control and the No Template Control (NTC).
3. The internal extraction control successfully performed when used, confirming the efficiency of RNA extraction and the absence of reaction inhibitors.

7- Mosquito collection and laboratory colony creation

Generation Details: Egg swarms were collected from a drainage area in Karbala Governorate using a long-handled scoop and placed in a covered plastic container. These were then transported to the laboratory and placed in plastic tanks filled with chlorine-free water. After hatching, the larvae were fed finely ground and sifted wheat bran daily in each tank during the first larval stages (first and second in stars). This amount was increased in the later stages (third and fourth in stars) to prevent deterioration of the rearing water quality and support larval growth and development [19]. To obtain a pure and continuous culture, the newly formed pupae were transferred to small plastic cups, which were then placed inside cube-shaped wooden cages. A glass Petri dish was placed in the center of each cage. After the adult insects emerged, and to feed them (males and females) and provide them with the energy necessary for flight and reproduction, Petri dishes containing a 10% sugar solution were placed inside the cages to feed the females, and flowering plants to feed the males and facilitate mating, according to methods used in rearing *Culex* mosquito colonies in the laboratory[20]. The rearing cages were monitored regularly to ensure the maintenance of suitable environmental conditions and the health of the insect colony throughout the study period. Because the *Culex molestus* strain exhibits self-reproduction, [21] a small plastic container filled with chlorine-free water was placed inside the cage to prepare it for egg-laying. The egg-laying vessels were then transferred



with a small brush, taking care not to disturb them to avoid disintegration, to freshwater containers filled with larval food. These vessels were monitored until the emergence of adult insects. Samples were taken from the fourth-generation larvae and from the third generation of adult insects. It was identified using the taxonomic keys of the mosquito family Culicidae, with the help of the supervisor, and was found to belong to the species *Culex molestus*. The insects were reared and their colonies were maintained under laboratory conditions at a temperature of 28 ± 2 °C, relative humidity of $55 \pm 5\%$, and a photoperiod of 12:12 hours (light:dark), which are common and suitable conditions for rearing *Culex* mosquitoes and maintaining their laboratory colonies [19, 22].

8- Rotavirus experimental groups

Three independent experimental groups were assigned to the rotavirus pathway, with 10 individuals per group, along with appropriate controls.

Group	Exposure Source	Duration of Exposure to Positive Source	Recipient Medium	Recipient Contact Duration
A1	Rotavirus A-positive fecal sample (molecularly confirmed)	1 hour	Molecularly confirmed Rotavirus A-negative fecal sample	8 hours
B1	Rotavirus A-positive fecal sample (molecularly confirmed)	4 hours	Molecularly confirmed Rotavirus A-negative fecal sample	8 hours
C1	Rotavirus A-positive fecal sample (molecularly confirmed)	24 hours	Molecularly confirmed Rotavirus A-negative fecal sample	8 hours
Control	No exposure to a viral source	—	Molecularly confirmed Rotavirus A-negative fecal sample	According to the control protocol

9- Implementation of the Mechanical Transport Experiment

Pupae were isolated from the laboratory colony and randomly distributed into experimental groups of 10 individuals each. They were then placed in rearing cages measuring 50×50×50 cm. After adult emergence, the necessary feeding sources were provided until the start of the experiment. Rotavirus-positive fecal samples, confirmed by



RT-qPCR, were used as viral sources. These samples were placed in Petri dishes inside the mosquito cages, and females were allowed to land and come into direct contact with the positive fecal medium. The exposure period lasted for 1 hour for group A1, 4 hours for group B1, and 24 hours for group C1. After the exposure period, the positive dishes were removed and replaced with new dishes containing partially negative fecal samples. A 10% sucrose solution was added to the receiving medium as a feeding and landing stimulus. The insects were left in direct contact with the receiving medium for 8 consecutive hours, then representative samples were collected from the receiving dishes in a sterile manner, coded and stored in refrigerated tubes until extraction and molecular testing using RT-qPCR were performed to detect the transmitted rotavirus RNA.

10-Control Groups

The design included a negative control group of virus-negative feces to verify the absence of contamination or spontaneous transmission, a control group of clean medium or distilled water to verify the integrity of the assay procedures, and a positive control group for rotavirus PCR consisting of a molecularly confirmed positive sample to verify the efficiency of the RT-qPCR assay. The study tables show that the number of samples in each of these groups was 10. A negative control group consisting of 10 female mosquitoes that had not been exposed to any viral source was also assigned. These mosquitoes were placed on clean fecal samples and subjected to the same molecular procedures and assays used for the experimental groups to verify the absence of contamination during the experimental stages.

Statistical Analysis

Data were analyzed using SPSS version 27. Continuous variables were expressed as mean $\pm$ SD. One-way ANOVA, Tukey's post hoc test and Pearson's correlation were used, with $P < 0.05$ considered statistically significant. Data obtained from rapid tests and RT-qPCR were entered into Microsoft Excel 2021 for data organization. Descriptive statistics were used to calculate frequencies, percentages, the mean, the standard deviation, the median, and the first and third quartiles (Q1 and Q3) when analyzing Ct values.



Results

Initial Rapid Cassette Test

The initial immunological and diagnostic assessment using the rapid cassette test revealed a 100% absolute positivity rate for all targeted samples. At total of seventy-five stool samples showed a clear positive reaction for rotavirus surface antigens, as detailed in Table 1.

Table 1: Rapid Cassette Test for Initial Detection of Rotavirus in Samples

Virus	Number of Samples Tested	Positive Samples	Negative Samples	Positivity Rate	95% CI
Rotavirus A	75	75	0	100%	95.2–100%

Molecular and Evaluation of the Confirmatory Test (RT-qPCR) for Rotavirus A

The results of molecular detection using (RT-qPCR) showed a significant decrease in the number and percentage of positive samples compared to those obtained from rapid immunoassays, highlighting the high selectivity and precise molecular screening capabilities. the molecular technique revealed viral RNA positivity in only 41 samples, achieving a rate of 54.7%, while 45.3% of the samples were positive by rapid testing but molecularly negative, as detailed in the cross-statistical comparison table) 2.(

Table 2: Reciprocal statistical comparison between rapid test results and RT-qPCR technique for rotavirus A (n = 75)

Rapid Test Result	RT-qPCR Positive, n (%)	RT-qPCR Negative, n (%)	Total, n (%)
Positive	41 (54.7%)	34 (45.3%)	75 (100%)
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	41 (54.7%)	34 (45.3%)	75 (100%)

To document the diagnostic efficiency of the Rapid Cassette Test and determine its selectivity in detecting rotavirus compared to RT-qPCR, the gold standard for confirmatory



testing, receiver operating characteristic (ROC) curve analysis was employed. Figure (1) shows the performance of both tests and the area achieved under the curve (AUC), which accurately translates the kinetic gap between immunological monitoring and effective molecular confirmation.

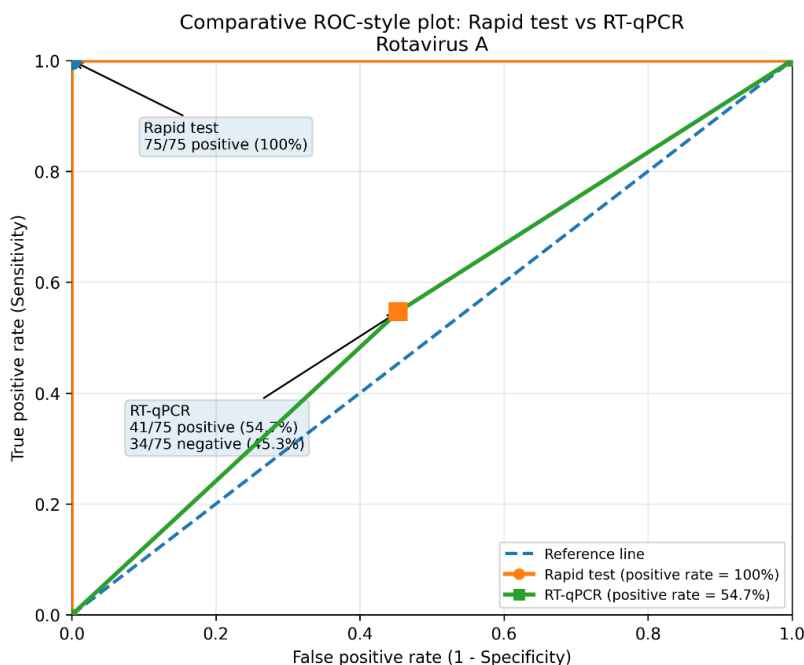


Figure 1: Comparative ROC curve of the performance of the rapid test and RT-qPCR technique in detecting rotavirus.

Quantitative Analysis and Classification of Rotavirus Viral Load (Ct-values)

The results of quantitative molecular amplification of rotavirus-positive samples ($n = 41$) showed a marked variation in the periodic threshold (Ct) values. Cycles ranged from a minimum of 16.8, indicating a very high viral load, to a maximum of 37.8, as detailed in the statistical description documented in Table 3.



Table 3: Comparative Statistical Description of Ct Values in Molecularly Positive Rotavirus Samples

Parameter	Value
Number of positive samples	41
Minimum Ct value	16.8
Maximum Ct value	37.8
Mean	27.63
Standard deviation (SD)	5.81
Median	27.5
First quartile (Q1)	23.1
Third quartile (Q3)	32.0

To quantify viral loads, positive samples were distributed into three main categories based on Ct values and the standard formula for viral RNA copy number (copies/ μL) as shown in Table 4

Table 4: Classification and Distribution of Viral Load in Rotavirus Samples According to Periodic Threshold (Ct) Values.

Ct Category	No. of Samples	Percentage	Viral Load (copies/ μL)	Viral Load Description
Ct < 25	14	34.1%	$>1 \times 10^4$	High viral load
Ct 25–30	12	29.3%	$1 \times 10^3 - 1 \times 10^4$	Moderate viral load
Ct > 30	15	36.6%	$<1 \times 10^3$	Low viral load
Total	41	100%	—	—

The figure 2 showed the amplification curves of 41 rotavirus-positive samples (red lines) alongside the positive control (blue line) and the negative control (NTC) (green line). All positive samples exhibited typical sigmoid amplification curves exceeding the threshold line (Threshold = 0.05), confirming the presence of viral RNA in the tested samples. Ct values ranged from approximately 18 to 38 cycles, reflecting the varying viral loads among the samples; lower Ct values indicate higher viral loads, while higher values indicate lower viral loads. The positive control showed early and significant amplification, while the negative control did not register any amplification throughout the reaction cycles, confirming the absence of contamination in the reaction and the validity of the molecular assay results.

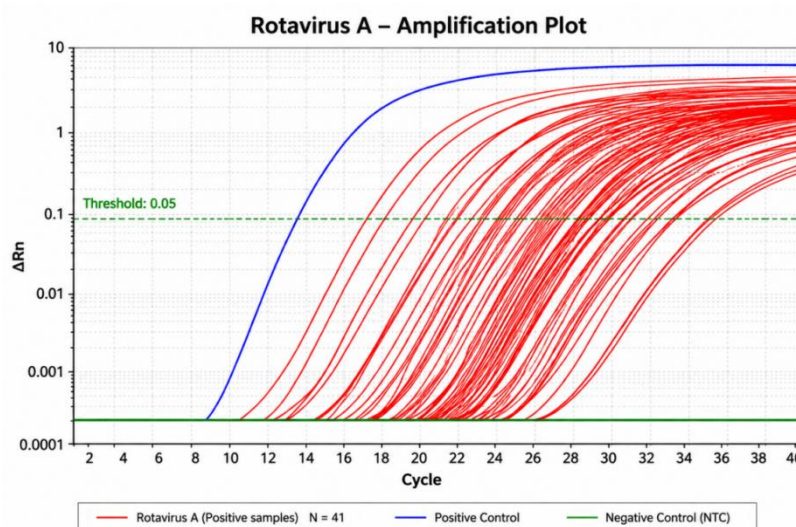


Figure 2: Amplification Plot of Rotavirus A using RT-qPCR technology in positive clinical samples.

Mechanical transmission of rotavirus A by female mosquitoes

RT-qPCR results showed successful detection of rotavirus A RNA in all clean fecal dishes exposed to female *Culex molestus* mosquitoes after contact with positive fecal sources. All three exposure treatments yielded positive results, while the unexposed control group remained negative and showed no amplification. Table (5).

Table 5: RT-qPCR results after mechanical transmission of rotavirus A

Group	Duration of Mosquito Exposure to Positive Source	Mean Ct $\pm$ SD	Viral Load (copies/ μ L)	RT-qPCR Result
A1	1 hour	35.84 $\pm$ 0.41	1.1 $\times$ 10 ¹	Positive
B1	4 hours	31.72 $\pm$ 0.36	1.7 $\times$ 10 ²	Positive
C1	24 hours	27.48 $\pm$ 0.28	3.3 $\times$ 10 ³	Positive
Control	Unexposed fecal sample	No Ct	Not detected	Negative

Ct values gradually decreased from 35.84 after 1 hour to 31.72 after 4 hours, then to 27.48 after 24 hours, concurrently with an increase in the estimated viral load from 1.1 $\times$ 10¹ to 3.3 $\times$ 10³.

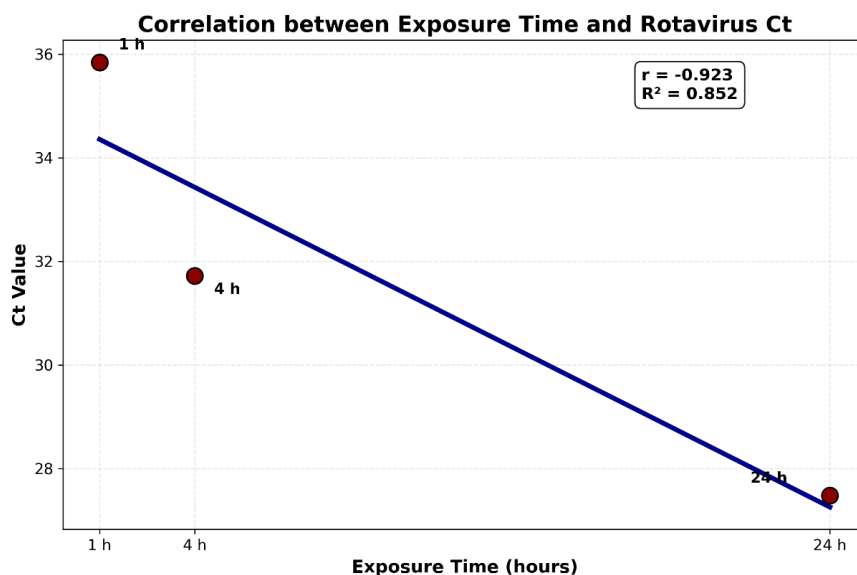


Figure 3: The relationship between the time of exposure of *Culex molestus* mosquitoes to contaminated fecal material and the Ct values of rotavirus detected by RT-qPCR.

Change in Transmitted Viral Load with Exposure Duration

The estimated mechanically transmitted viral load increased with increasing exposure duration, reaching $10^1 \times 1.1$ copies/ μ L after 1 hour, then rising to $10^2 \times 1.7$ copies/ μ L after 4 hours, and reaching $10^3 \times 3.3$ copies/ μ L after 24 hours.

Table 6: Change in Transmitted Viral Load with Exposure Duration

Exposure Duration	Transferred Viral Load (copies/ μ L)	Interpretation
1 hour	1.1×10^1	Low viral transfer after short exposure
4 hours	1.7×10^2	Marked increase in transferred viral RNA
24 hours	3.3×10^3	Highest recovered viral load

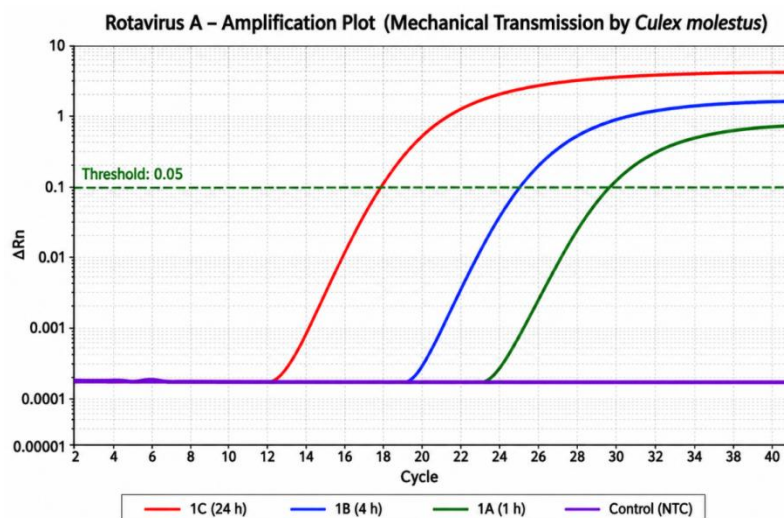


Figure 4: Comparison of amplification curves for rotavirus after mechanical transmission by female *Culex molestus* mosquitoes during different exposure periods.

Evaluation of Amplification Plots

Kinematic analysis of the molecular amplification plots for all positive samples ($n = 41$) showed typical sigmoidal curves with integrated phases, clearly exceeding the defined threshold reference line (Threshold = 0.05) without any interference or false background signals. The positive control exhibited stable early amplification, while the target-free negative control (NTC) maintained a flat baseline with no amplification signal throughout the 40 cycles, confirming the absolute reliability of the reaction and the absence of cross-contamination in the laboratory environment.

Comparison between Original and Mechanically Transmitted Samples

When comparing positive original samples with mechanically transmitted samples, the mean Ct of the original samples was 5.81 ± 27.63 , with an estimated viral load of approximately $10^4 \times 1.0$ copies/ μL . After mechanical transmission, the samples recorded Ct values of 0.41 ± 35.84 after 1 hour, 0.36 ± 31.72 after 4 hours, and 0.28 ± 27.48 after 24 hours. The ΔCt gap decreased from +8.21 after 1 hour to +4.09 after 4 hours, then reached -0.15 after 24 hours, with the recovery rate increasing from 0.11% to 1.7% and then to 33.0%.



Table 7: Comparison of Original and Mechanically Transmitted Rotavirus A Samples

Parameter	Original Samples (Rotavirus A)	Mechanical Transmission		
		(1 Hour)	(4 Hours)	(24 Hours)
No. of samples	41	1	1	1
Mean Ct $\pm$ SD	27.63 $\pm$ 5.81	35.84 $\pm$ 0.41	31.72 $\pm$ 0.36	27.48 $\pm$ 0.28
Viral load (copies/ μ L)	$\approx 1.0 \times 10^4$	1.1×10^1	1.7×10^2	3.3×10^3
RT-qPCR result	Positive	Positive	Positive	Positive
Δ Ct relative to original	—	+8.21	+4.09	−0.15
Viral load relative to the original	100%	0.11%	1.7%	33.0%
Evidence of transmission	—	Confirmed transmission	Confirmed transmission	Confirmed transmission

Molecular Recovery Rate After Mechanical Transfer

The results showed that the recovery rate of rotavirus A RNA increased significantly with increasing exposure time; it reached 0.11% after 1 hour, then 1.7% after 4 hours, and finally 33.0% after 24 hours.

Table 8: Rotavirus A RNA Recovery Rate After Mechanical Transfer

Exposure Duration	Δ Ct Relative to the Original Source	Molecular Recovery Rate
1 hour	+ 8.21	0.11%
4 hours	+ 4.09	1.7%
24 hours	− 0.15	33.0%

Statistical Analysis of the Effect of Exposure Duration

ANOVA showed a highly significant effect of exposure duration on rotavirus Ct values after mechanical transmission; $F = 112.7$ at $P < 0.001$. Post-hoc comparisons using Tukey's test also showed significant differences between all exposure durations: 1 with 4 hours, 1 with 24 hours, and 4 with 24 hours.



Table 9: ANOVA of the Effect of Exposure Duration on Rotavirus A Ct Values

Source of Variation	F-value	P-value	Significance
Exposure duration	112.7	<0.001	Highly significant

Table 10: Results of Tukey's post-hoc comparisons between exposure durations

Comparison	Mean Ct Difference	Adjusted P-value
1 hour with 4 hours	– 4.12	<0.001**
1 hour with 24 hours	– 8.36	<0.001**
4 hours with 24 hours	– 4.24	<0.001**

**** highly Significant**

Relationship between Exposure Duration and Ct Values

Correlation ship analysis showed a very strong inverse relationship between exposure duration and Ct values; Pearson's correlation coefficient $r = -0.923$, and the coefficient of determination $R^2 = 0.852$, at $P < 0.001$.

Table 11: Relationship between Exposure Duration and Molecular Detection Results for Rotavirus A

Virus	Correlation Coefficient (r)	Coefficient of Determination (R^2)	P-value	Interpretation
Rotavirus A	–0.923	0.852	<0.001	Strong inverse correlation between exposure duration and Ct value

Relationship between original Ct values and Ct values after transfer

The correlation analysis between original Ct values and Ct values after transfer showed a strong and significant positive relationship for rotavirus A, with the correlation coefficient $r = 0.94$ at $P < 0.001$.



Laboratory Control

The positive control for the Rotavirus NSP3 RT-qPCR assay showed clear amplification with a Ct value of 24.6, while the negative extraction control and NTC remained unamplified and registered No Ct. The internal control was also acceptable.

Table 12: Laboratory Control for the Rotavirus A RT-qPCR Assay

Assay	Positive Control (Ct)	Negative Extraction Control	No-Template Control (NTC)	Internal Control	Run Validity
Rotavirus NSP3 RT-qPCR	24.6	No Ct	No Ct	Acceptable	Valid

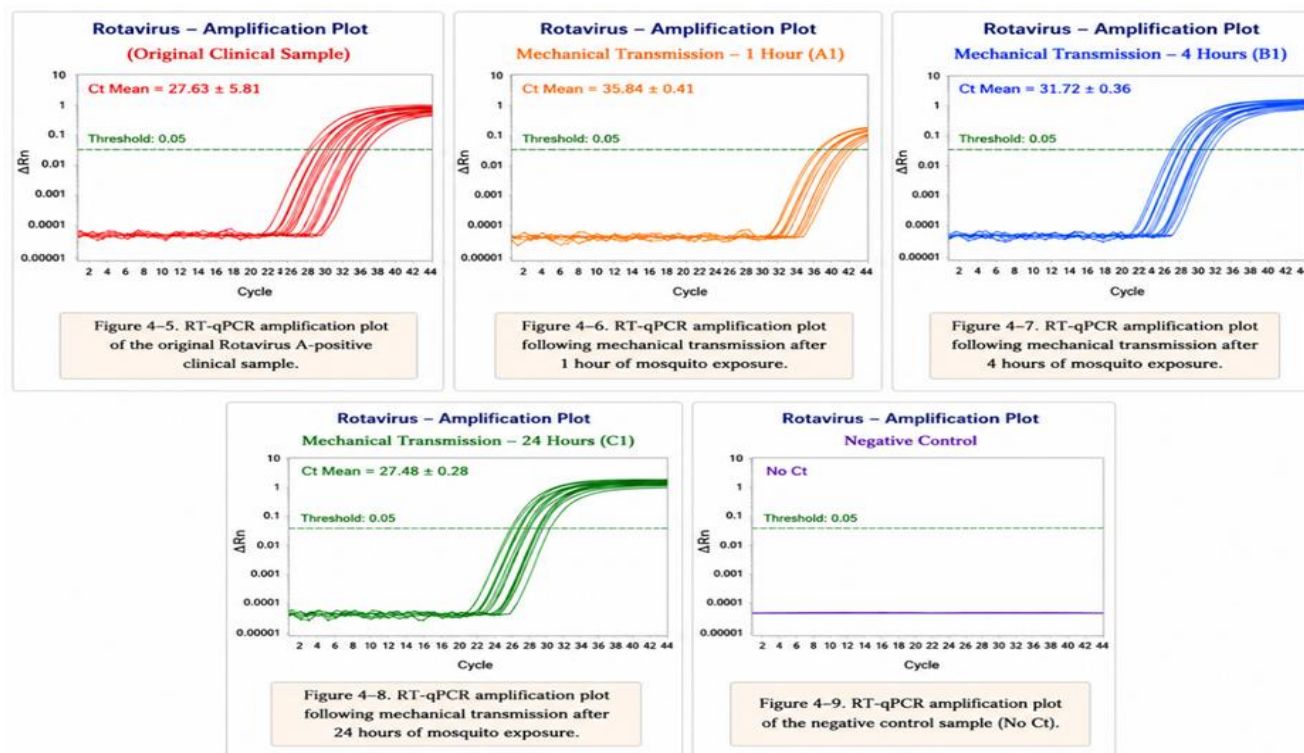


Figure 5: Comparative kinetic analysis of rotavirus amplification curves between original clinical samples and samples mechanically transmitted by *Culex molestus* females; a systematic gradual shift of threshold (Ct) values to the left (early crossing over) is observed in conjunction with the lengthening of exposure time from 1 hour to 24 hours, with the negative control (NTC) remaining unamplified.



Discussion

The current study provides clear molecular evidence of the mechanical transmission of rotavirus A RNA by female *Culex molestus* mosquitoes under controlled laboratory conditions. Viral RNA was detected in all clean fecal media exposed to mosquitoes after contact with the positive fecal source, while the control group remained negative. This finding is central to the study, as it demonstrates that mosquitoes can physically transfer viral genetic material from a contaminated source to a previously negative medium, without necessarily implying biological transmission or the transfer of live infectious virus. The rapid antigen test for rotavirus showed 100% positivity in all tested stool samples, while RT-qPCR confirmed rotavirus RNA in only 41 out of 75 samples, a molecular positivity rate of 54.7%. This discrepancy suggests that the rapid test, while useful as a primary screening tool, does not possess the same confirmatory accuracy as molecular detection, particularly in research studies that require direct confirmation of viral genetic material. The complete and absolute positivity (100%) recorded in this phase reflects the accuracy of the pre-clinical selection of samples. This aligns with studies comparing rapid antigen tests and RT-qPCR in rotavirus diagnosis, which indicated that rapid tests may differ from molecular results due to variations in analytical sensitivity, antigen concentration in the sample, stage of infection, and sample preservation and preparation methods [7,8,23].

The molecularly positive original samples showed wide variation in Ct values. This indicates a clear difference in viral load among clinical samples; lower Ct values reflect a higher viral RNA load, while higher values indicate lower loads or samples close to the detection limit. The amount of virus shed varies according to the stage of infection, symptom severity, age, immune status, vaccination, and time of sample collection. Studies by [7,8] have shown that the relationship between antigenic and molecular detection is influenced by viral shedding dynamics, and that RT-qPCR may detect RNA in time ranges or quantities that do not always correspond to rapid tests. The variation in Ct is not merely a technical one, but reflects a genuine biological difference in the amount of viral genetic material available in the source. This variation is crucial for interpreting the transmission



experiment, as a source with a high viral load provides a greater opportunity for the insect to ingest the viral material compared to a source with a low viral load.

The study demonstrated the successful transfer of rotavirus A RNA to clean fecal media via mosquitoes, with the negative control remaining free of amplification. This result supports the hypothesis that female *Culex molestus* are capable of mechanically carrying viral genetic material after contact with a contaminated fecal source and then depositing it in another medium. This interpretation aligns with the general concept of mechanical transmission, which does not require pathogen replication within the vector but rather relies on the physical carrying of the pathogen or its components on the legs, mouthparts, bristles, or the insect's external surface. Studies by [11,12,14] have indicated that insects may act as mechanical mediators in contaminated environments when they move between sources of contamination and clean recipients. The significance of this finding lies in its expansion of the concept of mechanical transmission to include urban mosquitoes adapted to environments close to humans, and not just traditional insects associated with feces, such as flies.

The results showed that increasing the duration of mosquito exposure to the contaminated source was associated with a significant decrease in Ct values and an increase in the estimated viral load. This pattern supports the hypothesis that mechanical transmission was a quantitative process influenced by contact time, rather than a random event. The longer the insect was with the contaminated source, the greater the opportunities for landing, walking, contact, and feeding, thus increasing the probability of capturing viral genetic material and depositing it in the host medium. The transmission efficiency is affected by contamination intensity, contact duration, insect surface characteristics, and the number of contacts with the source and host. Previous studies have shown that the efficiency of pathogen transmission is strongly influenced by the host-vector contact dynamics, duration of contact, severity of contamination, and frequency of vector interactions with contaminating sources [24]. Furthermore, the persistence of rotavirus in the environment and its ability to remain infectious on contaminated surfaces support the biological plausibility of indirect mechanical spread under favorable environmental



conditions [25]. This finding represents the core novelty of the study because it not only proves that transfer occurs, but also shows that the amount of transferred RNA gradually increases over time. Therefore, the phenomenon can be described as a molecular-mechanical transfer dependent on the duration of exposure to the contaminating source.

The comparison between the original source and transfer samples showed that the average original Ct was 5.81 ± 27.63 , while the Ct after transfer was 35.84 after 1 hour, 31.72 after 4 hours, and 27.48 after 24 hours. The Δ Ct difference also decreased from +8.21 after 1 hour to +4.09 after 4 hours, then reached -0.15 after 24 hours. The molecular recovery rate increased from 0.11% to 1.7% and then to 33.0%. The transfer after 1 hour was relatively weak, while the amount of recovered RNA after 24 hours became very close to the average of the original source. This supports the hypothesis that viral RNA gradually accumulates on the external surfaces of mosquito bodies or mouthparts during prolonged contact with contaminated materials [24]. Similar research observations have been recorded for mechanically transmitted enteric pathogens, where repeated contact with contaminated substrates and parts enhances the efficiency of microbial particle and viral material transmission [25]. Some recent systematic research confirms that the conventional RT-qPCR technique cannot distinguish between infectious and non-infectious viral particles, and that complementary confirmatory tests such as tissue culture or RT-qPCR for protein coat integrity are needed when studying viral infection [23,25]. Large increase in the recovery rate supports the idea of the gradual physical accumulation of viral material on the insect's body or external parts with the duration of exposure. However, the recovery rate should be interpreted with caution; This is a molecular recovery rate based on RT-qPCR, not a recovery rate of a live, infectious virus. The same study confirmed that relying on RT-qPCR only proves the presence of RNA and not viral viability or infectivity, thus necessitating future viral culture or infectivity assays.

Statistical analysis showed that exposure duration significantly affected rotavirus Ct values, with an F value of 112.7 at $P < 0.001$. Tukey's comparisons also revealed significant differences between 1 and 4 hours, 1 and 24 hours, and 4 and 24 hours, confirming that the differences between the time periods were not random. Correlation analysis also showed a



strong inverse relationship between exposure duration and Ct values, with Pearson's coefficient $r = -0.923$ and $R^2 = 0.852$ at $P < 0.001$. This indicates that increased exposure duration was associated with a decrease in Ct, i.e., an increase in the amount of viral RNA detected in the receiver medium. Since Ct values are inversely proportional to the amount of viral RNA detected, lower Ct values generally reflect higher concentrations of viral RNA [26]. Methodological approaches have shown that combining temporal gradients with multiple complementary analytical measures substantially increases the reliability of causal inference in laboratory transmission studies [27]. The strength of the evidence does not stem solely from the correlation coefficient, but rather from the combination of several elements: the stepwise time design, the consistent decrease in Ct, the concurrent increase in viral load, the negative control, and the presence of significant differences between the time periods. Therefore, the strongest conclusion is that exposure time is an important determinant of the efficiency of mechanical transport in this laboratory model.

The correlation analysis between original and post-transfer Ct values showed a strong and significant positive relationship, with $r = 0.94$ at $P < 0.001$. This result indicates that sources with higher viral loads maintained their tendency to produce higher molecular recovery after transfer, despite some quantification loss during the transfer process. This result is significant because it suggests that transfer was not entirely random but was related to the amount of RNA already present in the source. This aligns with the principles of mechano-transfer, which assume that the concentration of the pathogen in the source affects the amount of material that the insect can pick up and transfer. The efficiency of mechanical transfer of rotavirus A is influenced by two interrelated factors: the viral load in the source and the duration of mosquito exposure to that source. Therefore, environments with high fecal contamination may provide more favorable conditions for the mechanical pickup and transfer of viral material. Since RT-qPCR Ct values are inversely proportional to the concentration of target nucleic acids, samples with lower Ct values have higher viral RNA loads and therefore present more material available for mechanical acquisition and subsequent transfer [28]. These observations conform to the mechanics of pathogen transmission suggested by the theory, where transmission potential depends on combined effects of the load at origin, frequency and duration of exposure, as well as probability of successful transfer upon



contact with a recipient substrate [29]. Thus, the current findings demonstrate that two interdependent factors regulate the efficiency of rotavirus A molecular–mechanical transfer: viral RNA concentration within its contaminated source and time of mosquito exposure. Such interaction could also help to explain why the most heavily contaminated fecal environments are much more favorable for the acquisition and subsequent mechanical transmission of viral RNA by insects, although the results presented here showed that such molecular transfer is not equivalent to transmission of infective virus [30].

The positive control for the Rotavirus NSP3 RT-qPCR assay showed clear amplification with a Ct value of 24.6, while neither the negative extraction control nor the NTC showed any amplification, and the internal control was acceptable. This supports the reliability of the results and reduces the likelihood that the positive signals in the receiving media are due to accidental laboratory contamination. This result is consistent with the quality control requirements of qPCR assays, where positive and negative controls and the internal extraction control are essential to ensure reaction efficiency and the absence of contamination and inhibitors. Therefore, the lack of amplification in both the extraction control and the NTC provides strong evidence that the detected RT-qPCR signals originated from the experimental exposure pathway rather than cross-contamination during sample processing or amplification [31,32]. The success of the controls is a crucial element in interpreting the transfer results because the study deals with very small amounts of RNA after mechanical transfer, and any laboratory contamination could have led to misinterpretation. Therefore, the negative NTC and extraction control reinforce the conclusion that the positive signals are indeed related to the experimental exposure pathway.

The selection of female *Culex molestus* is of particular importance because this phylum is associated with urban environments and sites near humans and humid or polluted infrastructure, such as drainage sites and stagnant water. Studies such as [17,18,33] support the importance of studying this mosquito phylum in urban environments, especially when considering unconventional insect roles in polluted environments. These characteristics do not necessarily mean that the mosquito is an infectious vector for rotavirus, but they do



make testing its ability to mechanically carry viral material a legitimate scientific question, especially in environments where fecal contamination is combined with high insect density.

Novelty and Scientific Contribution of the Study

This study lies in its testing of an unconventional model for rotavirus A, namely the mechanical transmission of viral genetic material by urban mosquitoes, rather than focusing solely on the direct fecal-oral route or water and surface contamination. Furthermore, the study did not merely confirm the presence of RNA after transmission, but also investigated the effects of exposure duration, Ct values, estimated viral load, recovery rate, and the correlation between the source and transmission. The primary scientific contribution of this study is the presentation of an experimental model linking three elements: a highly excreted enterovirus, urban mosquitoes adapted to contaminated environments, and progressively longer exposure times to the viral source. These elements, taken together, make the study closer to a proof-of-concept study of molecular mechanical transmission. The results of the current study, supported by the scientific literature, indicate that female *Culex molestus* can mechanically transfer rotavirus A RNA from a positive fecal source to a previously negative fecal medium, and that the amount of transferred genetic material increases with exposure time to the contaminated source. This is supported by the Ct results, viral load, recovery rate, statistical analysis, and negative control results. Therefore, rotavirus A, due to its fecal excretion and relative environmental stability, may be more susceptible to molecular mechano-transmission in contaminated environments compared to some other viruses. However, the study does not prove rotavirus transmission by mosquitoes, but rather accurately demonstrates the mechanical transfer of rotavirus A RNA under controlled laboratory conditions.

Limitations of Interpreting the Results

Despite the strength of the results, they must be interpreted within their methodological limitations. First, RT-qPCR detects viral RNA but does not prove the presence of a live, infectious virus. Second, the study did not specify the location of RNA transport on the



mosquito body, such as the legs, mouthparts, or outer surface. Third, the experiment measures the effect of exposure time to the contaminated source and does not directly measure how long the mosquito retains the virus after leaving the source. Fourth, laboratory conditions do not necessarily represent the full complexity of the natural environment. Therefore, the next step should include testing the viability of the transmitted active virus using cell culture or an infectivity assay, examining mosquito body parts separately, conducting an independent retention time experiment.

Conclusions:

The study demonstrated the possibility of mechanical transfer of rotavirus A genetic material (RNA) by female *Culex molestus* mosquitoes under controlled laboratory conditions. Viral RNA was transferred to previously negative fecal media after exposure to mosquitoes that had come into contact with positive sources, while the control group remained negative. RT-qPCR results showed that increasing the duration of mosquito exposure to the contaminated source led to an increase in the amount of viral RNA transferred. Ct values gradually decreased, and the estimated viral load and molecular recovery ratio increased with increasing exposure time, indicating that mechanical transfer is a quantitative process dependent on the duration of contact with the contaminated source. Statistical analysis confirmed a strong inverse relationship between exposure duration and Ct values, indicating that time is a key factor in the efficiency of mechanical transfer within the experimental model used.

The original samples positive for rotavirus A showed variation in viral load, with Ct values indicating that the efficiency of mechanical transmission may also be affected by the viral load of the original source. The study provides experimental evidence for the potential of *Culex molestus* to act as a mechanical vector for the transfer of rotavirus A genetic material under conditions of direct contact with contaminated fecal sources, without proving its role as a biological vector for the virus. The study results demonstrate the transfer of viral genetic material (RNA). However, they do not prove the transmission of a live, infectious virus. Therefore, future studies involving viral culture and viability tests are needed to assess the potential for transmission of an active infectious virus.



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