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Assessment of the Mechanical Transmission Potential of Coxsackievirus RNA by Female *Culex molestus* Mosquitoes under Controlled Laboratory Conditions

Rusul Abdulnabi Wali[1*], Rafid Abbas Ali[1], Saif Jabbar Yasir[2]

[1]Department of Biology, College of Education for Pure Sciences, University of Karbala, Karbala, Iraq
[2]Department of Medical Microbiology, College of Medicine, University of Kufa, Najaf, Iraq

Abstract

Introduction: Coxsackieviruses are medically important enteroviruses transmitted mainly by the fecal–oral route. Although mosquitoes are studied as biological vectors of arboviruses, their role in the mechanical transmission of enteroviruses remains unclear. The aim of this study was to evaluate the susceptibility of female Culex molestus mosquitoes to mechanical transmission of Coxsackievirus genetic material, and the effect of exposure duration on the amount of viral RNA transferred.

Methods: Fifty blood and stool samples were collected from participants. IgM was screened by rapid immunoassay and stool viral RNA by RT-qPCR; positive samples served as viral sources. Female C. molestus were exposed to positive fecal sources for 1, 4, or 24 hours, then transferred to negative fecal media for 8 hours; recipient-media RNA was quantified by RT-qPCR.

Results: All 50 samples were IgM-positive; RT-qPCR confirmed Coxsackievirus RNA in 22/50 (44.0%), with Ct 19.2–37.6 (mean 28.75 ± 5.31). Transfer was detected after all three periods, with mean Ct decreasing from 36.92 to 33.61 to 29.84 and molecular recovery rising from 0.09% to 0.82% to 10.8% at 1, 4, and 24 hours. ANOVA showed highly significant differences ($F = 96.3$, $P < 0.001$), with a strong inverse correlation between exposure duration and Ct ($r = -0.934$, $R^2 = 0.872$, $P < 0.001$). Controls showed no amplification.

Conclusion: Female C. molestus can mechanically transfer Coxsackievirus RNA from a positive fecal source to a negative medium under laboratory conditions, with transfer increasing with exposure time. RT-qPCR confirms RNA transfer but not infectivity; further studies of viability and field relevance are warranted.

Keywords: Coxsackievirus, Viral RNA, *Culex molestus*, Mosquitoes, Mechanical transmission, Ct value, Duration of exposure, RT-qPCR.

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Introduction

Infectious diseases associated with environmental pollution are, of the public health system challenges in rich cities and developing communities suffering from failing sanitation systems and unsustainable management of stagnant water based organic wastes. These circumstances may provide optimal ecological conditions for insect growth, exposing insects more abundantly to human waste or sources of microbial contamination and causing them to transfer repeatedly from contaminated environments to sites linked with human activity. The changed dynamics of pathogen transmission associated with insect vectors, linked not only to natural evolution but also to environmental factors (ecosystem changes), uncontrolled urbanization and the deterioration of sanitation systems, impose a need for a reevaluation of the traditional and non-traditional roles played by insects in these processes (1,2) Enteric viruses are particularly important here due to their fecal-oral route of transmission and the capacity for residues of their genetic material to persist in mediums such as sewage, polluted water, and many environmental surfaces. In addition, many of these viruses are non-enveloped and thus somewhat environmentally stable when compared to enveloped viruses. This allows them to survive in polluted habitats for a sufficient duration that indirect transmission by water, fomites, or organisms which travel between environmental reservoirs and susceptible faunal hosts is possible. Boone and Gerba (3) highlighted the importance of environmental stability and the transmission dynamics of enteric viruses, while Graczyk et al. (4) explored the relationship between their survival in contaminated ecosystems and the potential for human-associated vectors to contribute to their mechanical transmission.

Coxsackievirus is a medically important enterovirus belonging to the genus Enterovirus within the family *Picornaviridae*. It is a small, non-enveloped virus with a positive-sense, single-stranded RNA genome. Its structural and molecular characteristics contribute to its interaction with host cells and its relative stability in various environments. Coxsackieviruses are associated with a wide range of clinical manifestations, from mild or asymptomatic infections to more severe illnesses, including some forms of hand, foot, and mouth disease, aseptic meningitis, myocarditis and pericarditis, as well as some

*Corresponding Author: Rusul Abdulnabi Wali, Email: resul.a@s.uokerbala.edu.iq

neurological and systemic complications that depend on the viral genotype, the age of the infected individual, and their immunological status. (5, 6) Coxsackieviruses are primarily transmitted via the fecal-oral route, with direct contact, respiratory secretions, and contaminated surfaces and objects potentially contributing to the transmission of some viral serotypes. The shedding of the virus or its genetic material in feces is an important factor in the continuation of the transmission cycle, particularly in environments with poor sanitation. Moreover, the detection of viral RNA may still be observed during different phases of the infectious process (7, 8), which suggests that fecal samples may also represent a valuable tool for molecular surveillance of enteroviruses.

In environments with overlapping sources of fecal contamination and insect breeding sites, assessing the possible role of insects in indirect transmission of some pathogens will gain importance. Depending on the kind of relationship between pathogen and vector, insect-borne infections can be further categorized into two main types: biological transmission and mechanical transmission. Biological transmission means that the pathogen should be taken into blood circulation system of vector i.e. it must a penetrate through anatomical & cellular barriers. This may involve the pathogen multiplying and dispersing at some particular tissue level before being capable of being transmitted to a new host. In contrast, mechanical transmission does not involve multiplication or development of the pathogen within the digestive tract of the insect. Rather, such transmission is dependent on the mechanical transport of the pathogens or their elements with external body parts, including legs, hairs and mouthparts (10), or by intermittent transfer resulting from repeated contact between the contaminating source and then recipient environment (9,10).

Traditional studies of mosquitoes have largely focused on their role as biological vectors of arboviruses. Transmission efficiency in this case depends on the virus's ability to infect insect tissues, cross the midgut barrier, spread within the body, and reach the salivary glands. This process is related to what is known as vector competence and the extrinsic incubation period (11, 12). However, focusing on biological competence alone may not be sufficient to explain all forms of interaction between insects and pathogens found in contaminated environments, especially in the case of pathogens that do not need to complete an evolutionary cycle within the insect to be physically transferred from one source to another. In this context, studies on mechanical vectors have shown that insects that live in symbiosis with humans can contribute to carrying pathogens from contaminated sources to other surfaces or media as a result of direct and repeated contact, and that the efficiency of this process is affected by the characteristics of the pathogen, the nature of the insect's body surface, the duration of contact, the microbial load in the source, and the surrounding environmental conditions. (2,10,13) The epidemiological importance of this mode of transmission stems from the fact that it does not require a complex specialized relationship between the pathogen and the vector, but rather it can depend on a series of events of contamination, adhesion, and physical transfer between sources and environmental receptors. Among the medically important mosquito species, *Culex molestus* is a widespread group associated with urban, enclosed, and semi-enclosed environments, including basements, underground facilities, and some environments related to sewage systems and organic-rich water. In addition, this form also has various behavioral and biological traits that make it distinct from other Culex mosquitoes, namely the fact that it tries to mate in small spaces and that a least some populations are superbly suited to urban areas. (14, 15)

While Culex molestus is commonly found in urban habitats and organic rich sites, this raises the scientific question about its potential incidental mechanistic vector role in disseminating genetic material from some enteroviruses by direct contact with feces contaminated sources. This question is of great importance considering the rapid transit time of a mosquito from exposure to a contaminated source to another environment, and rationale can be made that what amount of mechanically transmitted viral material could depend on how long they engage in this behavior and the initial quantity of viral genetic material available. The need to re-evaluate the possible role of mechanical transmission by Diptera in poorly sanitized urban habitats in light of both structural and environmental determinants is underscored by findings from a number of recent studies that have investigated these subjects worldwide (2, 16).

Methodologically, improvements in molecular detection methods have facilitated transmission studies and the tracking of viral RNA in clinical and environmental samples. In this study, we showed RT-qPCR techniques that are sensitive, specific and provide quantitative or semi-quantitative comparisons between samples if the extraction and amplification conditions were controlled. The cyclic threshold (Ct) value is a measure of the detectable template molecules, correlated inversely with the amount of target RNA in a standard experimental protocol. Thus, if the dynamics of viral RNA transfer between experimental groups are to be estimated and if the impact of exposure time to the contaminating source on detectable RNA in a recipient is to be compared, Ct values can be tracked (17).

While some advances have been made in the molecular and classical epidemiology of Coxsackievirus, its conventional transmission routes, and molecular diagnosis, far fewer data exist concerning the potential for mechanical transmission by mosquitoes and the genome of Coxsackievirus than there are for biological transmission of arthropod-borne viruses. In addition to this, the detection of viral RNA attached to an insect does not prove that transmission is taking place; for experimental validation it needs a powerfully designed experiment with a known positive control, negative traces, clean receptors and defined exposure periods along with some evidence of molecular signaling at those receptors after contact by exposed insects. Studies of insect vectors have historically

focused on the morphology of their mouthparts in order to infer the ability of an insect to transmit viruses or trypanosomes (9). Thus, the present study was designed to investigate whether female Culex molestus mosquitoes were capable of mechanically transmitting Coxsackievirus genetic material under experimental laboratory conditions and assess how long infected insects would need to be exposed to the contaminated fecal source before any viral RNA could be detected in a clean medium receiving transfer from such an insect using RT-qPCR technology. This is important because it tests the hypothesis of non-traditional transmission by mosquitoes adapted for urban environments, in terms of both time and quantity of genetic material transmitted, without confusing detection of viral RNA with evidence that an infective virus was present.

Materials and Methods

Study Design and Experimental Methodology

We performed a controlled laboratory experiment to assess mechanical transmission of Coxsackievirus RNA by female Culex molestus mosquitoes. The study consisted of two interrelated stages: firstly, serological investigation of IgM antibodies to Coxsackievirus in blood samples followed by molecular detection of viral RNA from stool samples corresponding to the same cases by quantitative reverse transcription polymerase chain reaction (RT-qPCR). The second phase included the selection of molecularly positive stool samples and their use as a source of viral contamination in the mechanical transmission experiment by female mosquitoes. The transfer experiment involved exposing homogeneous groups of female mosquitoes to fecal sources that were molecularly confirmed positive for Coxsackievirus, followed by transferring the insects to previously molecularly confirmed virus-negative fecal media. Three independent exposure periods to the positive source—1 hour, 4 hours, and 24 hours—were used, each followed by a fixed 8-hour exposure to the negative fecal medium. The receiving medium was then analyzed using RT-qPCR to detect the transferred viral RNA.

Collection of Clinical Study Samples

Fifty blood samples were collected from patients attending Al-Zahraa Maternity and Children's Hospital in Najaf Governorate between January 28, 2025, and April 28, 2025. The samples included patients of different ages and both sexes who presented with clinical symptoms consistent with enterovirus infection, as assessed by a specialist physician. Whole blood samples were used, or serum or plasma was separated, depending on the requirements of the rapid testing kit. Counter stool samples were also collected from the same cases, providing each case in the Coxsackievirus group with a corresponding blood sample for serological testing and a corresponding stool sample for molecular detection. The blood samples were used for initial detection of IgM antibodies, while the stool samples were used for viral RNA detection using RT-qPCR. The total number of stool samples for the Coxsackievirus group was 50.

Ethical Standards and Biosafety

Human samples were managed in compliance with ethical and institutional approvals described in the original study. Written informed consent was obtained from the participants or their guardians, and identification codes rather than names were used to ensure data confidentiality. Approval was obtained from the appropriate Scientific and Ethical Committee (reference number 4098) and ethical principles for research using human samples were observed, as noted in the letter. In summary, all blood and stool samples were considered infectious material and handled following Biosafety Level 2 (BSL-2) practices. Personal protective equipment and sterile, single-use instruments were used, and work surfaces were disinfected before and after procedures using 70% ethanol. Waste was disposed of according to biosafety procedures.

Rapid Serological Test for Coxsackievirus

The initial screening was performed using the Coxsackievirus B IgM Test Kit, Colloidal Gold Method, manufactured by BABIO/Jinan Babio Biotechnology, China, according to the manufacturer's instructions. This step was a preliminary screening for the qualitative detection of antivirus- IgM antibodies in whole blood, serum, or plasma, and is not a substitute for direct molecular detection of viral RNA. The samples, cassettes, and test solution were allowed to reach room temperature. The cassette was then opened immediately before use and placed on a flat surface. Approximately 10 µL of whole blood, serum, or plasma was added to the sample well, followed by two drops of test solution, totaling approximately 70–100 µL. The result was read after 15 minutes; readings after 20 minutes were not considered valid. A result was considered negative if only the C control line appeared, and tentatively positive if both C and T lines appeared. The test was considered invalid if no control line was present.

Preparation of Stool Samples for Molecular Analysis

Stool samples for the molecular detection of Coxsackievirus RNA were prepared as a fecal suspension with an approximate concentration of 10% w/v in sterile PBS. The suspension was thoroughly mixed using a Vortex device and then centrifuged to remove coarse particles. The supernatant was used for RNA extraction. This step was employed to minimize the effect of naturally occurring inhibitors in stool and to improve the efficiency of the RT-qPCR reaction.

Viral RNA Extraction

Viral RNA was extracted using the innuPREP Virus DNA/RNA Kit-IPC16 from AJ Innuscreen/Analytik Jena, Germany. 200 µL of the prepared superlayer from the fecal suspension was used for each sample, and carrier RNA, proteinase K, and internal extraction control were added according to the manufacturer's instructions. Extraction was performed using the InnuPure C16 system or the system available according to the kit's protocol. The extracted RNA was collected in a final extraction volume

of 50–100 µL according to the manufacturer's protocol and then used immediately in RT-qPCR or stored at −80°C until the assay to minimize degradation.

Molecular Detection of Coxsackievirus Using RT-qPCR

A single-step RT-qPCR technique based on a TaqMan probe was used to detect viral RNA. Since Coxsackievirus belongs to the Enterovirus genus, the test targeted the conserved 5′-UTR region of the viral genome.

The expected amplification output length was approximately 192 base pairs, and the probe targeted an internal region confined between the EVF and EVR initiators (Table 1).

Preparation of the RT-qPCR reaction mix

The reaction was prepared with a final volume of 20 µL per sample, using the One-Step RT-qPCR Probe Master Mix. The reaction consisted of 10 µL of 2 × Master Mix, a forward primer with a final concentration of 0.6 µM, a reverse primer with a final concentration of 0.6 µM, a TaqMan probe with a final concentration of 0.25 µM, enzyme mix according to the manufacturer's instructions, and 5 µL of extracted RNA. The volume was then supplemented to 20 µL using nuclease-free water.

Thermal Amplification Program

The reaction was carried out according to the following thermal program: Reverse transcription at 48°C for 30 minutes for one cycle, then initial denaturation at 95°C for 10 minutes, followed by 45 cycles that included denaturation at 95°C for 15 seconds, then binding, elongation and fluorescence reading at 60°C for 60 seconds.

Quality Control and Interpretation of RT-qPCR Results

Each run included a positive control to verify the reactivity, primers, and probe; a negative extraction control to detect contamination during extraction; a non-formula-free (NTC) control to detect contamination of reaction components or water; and an internal amplification control to detect potential inhibitors in stool samples. Reactions were also performed repeatedly to minimize technical error and improve the reliability of measurements.

Mosquito Collection and Laboratory Colony Establishment

Egg swarms were collected from a drainage site in Karbala Governorate using a long-handled scoop and transported in covered plastic containers to the laboratory, where they were placed in tanks containing chlorine-free water. After hatching, the larvae were fed 0.2 grams daily of fine, sifted bran flour for the first and second larval stages, and this was increased to 0.5 grams daily for the third and fourth stages. The newly formed pupae were transferred to small cups within 50 × 50 × 50 cm rearing cages. After the adults emerged, the cages were supplied with cotton pieces soaked in a 10% sucrose solution, in addition to the flowers used for feeding the males. The colony was maintained, and the cages were cleaned and the food sources replenished regularly. Rearing continued until the third generation, then specimens of fourth-instar larvae and adults were identified using the taxonomic keys of the *Culicidae* family and the colony was maintained under constant laboratory conditions of 28 ± 2°C, 55 ± 5% relative humidity, and a 12-hour light: 12-hour dark photosystem**.**

Experimental Design for Mechanical Transmission of Coxsackievirus

Pupae were isolated from the laboratory colony and randomly assigned to experimental groups of 10 individuals each. After adult emergence, female mosquitoes were used in exposure experiments. The Coxsackievirus transfer pathway included three independent time treatments, along with appropriate control groups (Table 2).

Implementation of the Mechanical Transport Experiment

Previously confirmed high viral load fecal samples using RT-qPCR were used as the virus source. These samples were placed in Petri dishes within mosquito cages, and females were allowed to land and come into direct contact with the surface of the positive fecal medium. The exposure period lasted for 1 hour, 4 hours, or 24 hours, depending

Table 1. primers and probe that were used

Component	Type	The sequence 5′→3′	Target area
EVF/Up	Forward primer	GGCCCCTGAATGCGGCTAAT	5′-UTR
EVR/D w	Reverse primer	CACCGGATGGCCAATCCAA	5′-UTR
EV Probe/Pr	TaqMan probe	FAM-CGGACACCCAAAGTAGTCGGTTCCG-TAMRA	5′-UTR

Table 2. Experimental design to study the efficiency of mechanical transmission of Coxsackievirus by female *Culex molestus* mosquitoes under different exposure times

Group	Initial source	Duration of exposure to the positive source	Receiving medium	Duration of subsequent exposure
A2	Stool molecularly positive for coxsackie	1 hour	Molecularly negative stool	8 hours
B2	Stool molecularly positive for coxsackie	4 hours	Molecularly negative stool	8 hours
C2	Stool molecularly positive for coxsackie	24 hours	Molecularly negative stool	8 hours
Control	Negative medium not exposed to a viral source	—	Negative medium	According to the control protocol

The durations of exposure to Coxsackie were 1 hour, 4 hours and 24 hours, and all groups were then transferred to the clean medium for 8 hours.

on the group. At the end of the exposure period, the positive dishes were removed and replaced with new dishes containing fecal samples that were molecularly negative for the virus. A 10% sucrose solution was added to the receiving medium as a feeding and landing stimulus. The insects were allowed direct contact with the negative medium for 8 hours to allow for movement and contact of their legs and mouthparts with the receiving medium. After the 8-hour contact period, representative samples were collected from the receiving dishes using sterile methods, labeled, and stored in refrigerated microcentrifuge tubes until molecular analysis could be performed. A fecal suspension was then prepared and RNA was extracted using the same method as in the source samples, and examined using RT-qPCR to detect the presence of Coxsackievirus RNA and assess mechanical transport.

Control Groups

Analyses were performed using a virus-free feces negative control group to rule out potential contamination or spontaneity of transmission, a distilled water clean medium negative control group to verify the integrity of testing procedures, and a Coxsackievirus (PC-C) positive control group composed exclusively of molecularly confirmed PCR positivity for all samples to validate efficiency with RT-qPCR. The design tables show that the number of samples allocated to each of these groups was 10. A negative control group of 10 female mosquitoes that had not been exposed to any viral source was also allocated. These mosquitoes were placed on clean fecal samples and subjected to the same extraction and molecular testing procedures used for the experimental groups, with the aim of detecting any accidental contamination during the experiment.

Statistical Analysis

The raw data were organized using Microsoft Excel 2021, and the statistical analysis was performed using IBM SPSS Statistics version 26. Descriptive statistics were used to calculate frequencies, percentages, arithmetic means, standard deviations, median, and the first and third quartiles of Ct values. A statistical significance level of $P < 0.05$ was adopted.

Results

Rapid Test and Molecular Confirmation Results for Coxsackievirus

The rapid test results showed that all tested blood samples were initially positive for IgM antibodies against Coxsackievirus, with a 50/50 positivity rate (100%). However, the confirmatory molecular test (RT-qPCR) of the corresponding stool samples confirmed the presence of Coxsackievirus RNA in only 22 out of 50 samples, a molecular confirmation rate of 44.0%, while 28 samples were molecularly negative 56% (Table 3 and Figure 1).

Table 3. Comparison between Rapid Test and RT-qPCR for Coxsackievirus Detection

Rapid Test Result	RT-qPCR Positive n (%)	RT-qPCR Negative n (%)	Total n (%)
Positive	22 (44.0%)	28 (56.0%)	50 (100%)
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	22 (44.0%)	28 (56.0%)	50 (100%)

Results of Ct values and viral load in the original positive samples

The 22 molecularly positive samples showed a clear variation in Ct values, ranging from 19.2 to 37.6, with a mean of 28.75 ± 5.31 and a median of 28.95. This distribution indicates a difference in viral load intensity among patients, from high to low loads close to the detection limit (Table 4 and Figure 2).

Viral Load Classification in Original Samples

Based on Ct values, positive samples were classified into three categories. The low viral load category (Ct > 30) recorded the highest percentage at 45.4%, while the high viral load category (Ct < 25) and the intermediate viral load category (Ct 25–30) each recorded 27.3% (Table 5).

Results of Mechanical Transmission after Different Exposure Durations

RT-qPCR results showed the presence of Coxsackievirus RNA in all clean fecal media exposed to mosquitoes after contact with the positive source. Ct values gradually decreased from 36.92 ± 0.44 after 1 hour to 33.61 ± 0.38 after 4 hours, and then to 29.84 ± 0.31 after 24 hours, with the control group remaining negative (No Ct), (Table 6 and Figure 3).

Change in Transmitted Viral Load with Exposure Duration

The estimated transmissible viral load increased from 4.5×10^0 copies/µL after 1 hour to 4.1×10^1 copies/µL after 4 hours, and then to 5.4×10^2 copies/µL after 24 hours. This reflects an increase in the amount of mechanically transmitted viral RNA with increasing mosquito contact time with the contaminated source (Table 7 and Figure 3).

Comparison between Original and Mechanically Transported Samples

The average Ct in the original samples was 28.75 ± 5.31, while the mechanically transported samples recorded higher values: 36.92 ± 0.44 after 1 hour, 33.61 ± 0.38 after 4 hours, and 29.84 ± 0.31 after 24 hours. The difference between Ct and the original source decreased from +8.17 after 1 hour to +4.86 after 4 hours, and then to +1.09 after 24 hours, indicating that the recovered load approached that of the original source with increasing exposure time (Table 8 and Figure 4).

Molecular Recovery Rate after Mechanical Transfer

The molecular recovery rate increased from 0.09% after 1 hour to 0.82% after 4 hours, and then reached 10.8% after 24 hours. This result indicates that prolonging the mosquito's exposure to the contaminated source increased the efficiency of viral RNA capture and transfer to the clean

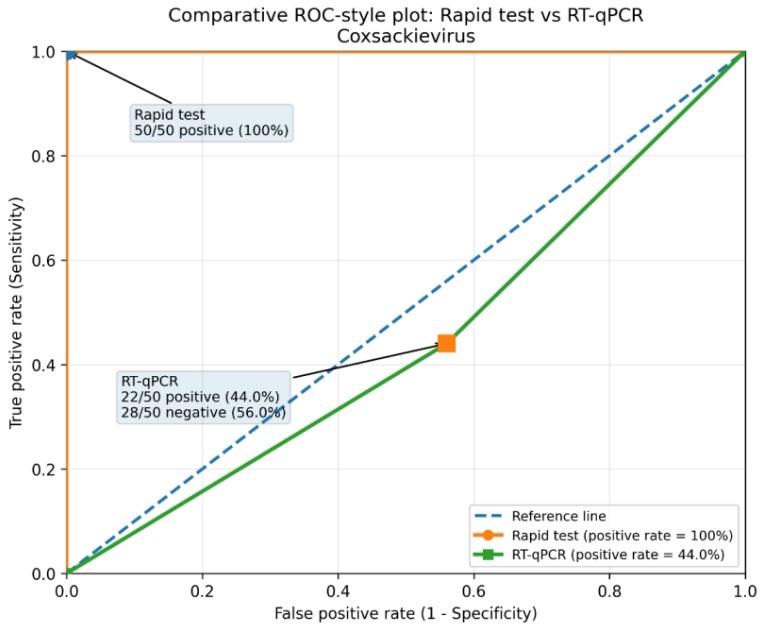

Figure 1. Comparative ROC curve of rapid test performance and RT-qPCR technology in detecting Coxsackievirus

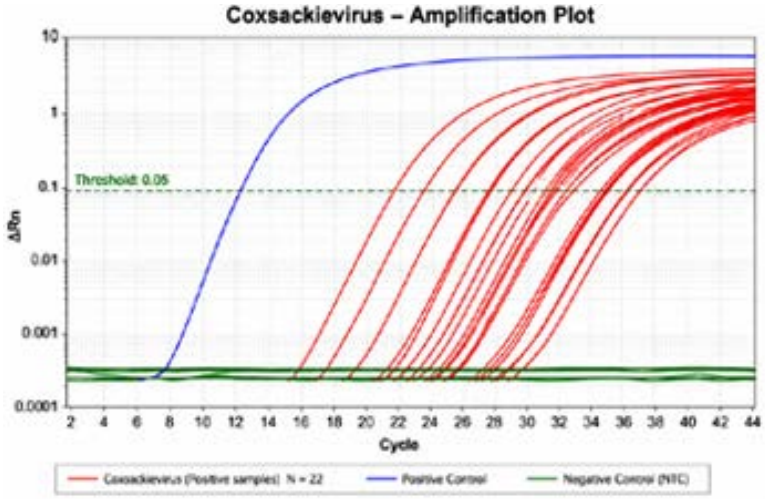

Figure 2. Amplification plot for molecular detection of Coxsackievirus using RT-qPCR technology

Table 4. Statistical description of Ct values in the molecularly positive Coxsackievirus samples

Parameter	Value
Number of positive samples (n)	22
Minimum Ct value	19.2
Maximum Ct value	37.6
Mean	28.75
Standard deviation (SD)	5.31
Median	28.95
First quartile (Q1)	25.1
Third quartile (Q3)	32.85

environment (Table 9).

Statistical Analysis of the Effect of Exposure Duration

One-way ANOVA showed a very significant effect of exposure duration on Ct values after mechanical transfer, with $F = 96.3$ at $P < 0.001$. This indicates that the duration of exposure to the contaminated source clearly affected the amount of viral RNA transferred (Table 10).

Relationship between Exposure Duration and Ct Values

Correlation analysis showed a very strong inverse relationship between exposure duration and Ct values, with a correlation coefficient $r = -0.934$ and a coefficient of determination $R^2 = 0.872$ at $P < 0.001$. This indicates that increased exposure duration was associated with decreased Ct and increased transmissible viral RNA (Table 11 and Figure 5).

Laboratory Control Results

The Coxsackievirus assay positive control had clear amplification at a Ct value of 25.1, while the negative extraction control and NTC control both displayed no evidence of amplification (No Ct value). These outcomes validate that the operation is a valid one, and there is no contamination of the reaction involved (Table 12).

Table 5. Viral Load Classification of Coxsackievirus Samples According to Ct Values

Ct Category	Number of Samples	Percentage (%)	Estimated Viral Load (copies/µL)	Viral Load Description
Ct < 25	6	27.3%	>5 × 10^3	High viral load
Ct 25–30	6	27.3%	5 × 10^2 – 5 × 10^3	Moderate viral load
Ct > 30	10	45.4%	<5 × 10^2	Low viral load
Total	22	100%	—	—

Table 6. RT-qPCR Results for Coxsackievirus RNA Detection after Mechanical Transmission

Plate	Exposure Time	RT-qPCR Result	Estimated Viral Load	Ct (Mean ± SD)
2A	1 hour	Weak positive	4.5 × 10^0	36.92 ± 0.44
2B	4 hours	Positive	4.1 × 10^1	33.61 ± 0.38
2C	24 hours	Positive	5.4 × 10^2	29.84 ± 0.31
Control	Not exposed	Negative	Not detected	No Ct

Table 7. Change in Transmitted Viral Load with Exposure Duration

Exposure Time	Transferred Viral Load (copies/µL)	Relative Change
1 hour	4.5 × 10^0	Lowest viral load
4 hours	4.1 × 10^1	Marked increase
24 hours	5.4 × 10^2	Highest viral load

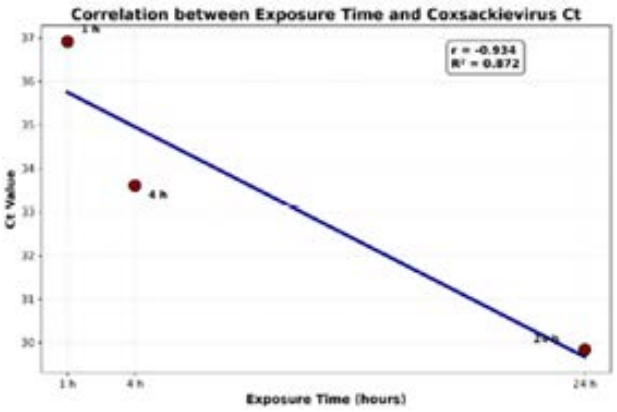

Figure 3. Mathematical correlation analysis between exposure duration and Ct values for mechanically transmitted Coxsackievirus; the consistent inverse linear regression line is observed, linking increasing time periods with decreasing cycle threshold (Ct) values

Discussion

This study demonstrated that, under controlled laboratory conditions, female Culex molestus were capable of acquiring and mechanically transferring Coxsackievirus genetic material from a positive fecal source to previously virus-negative fecal media. Positive RT-qPCR amplification was detected in all exposed treatments, whereas all unexposed control media remained negative. Furthermore, a clear temporal trend was observed, with the mean Ct value progressively decreasing from 36.92 ± 0.44 after 1 h of exposure to 33.61 ± 0.38 after 4 h and 29.84 ± 0.31 after 24 h, accompanied by a corresponding increase in the estimated viral RNA load. Collectively, these findings indicate that the transfer process was not merely a binary event (presence or absence of detectable RNA), but rather a quantitative phenomenon associated with prolonged contact between mosquitoes and the contaminated source. Similar observations have been reported for mechanically transmitted pathogens, where the amount of pathogen transferred is influenced by the intensity and duration of exposure to contaminated substrates rather than by pathogen replication within the arthropod vector (18).

The present findings are consistent with the general concept of mechanical transmission, in which an arthropod acquires pathogens or their genetic material from a contaminated source and subsequently transfers them to another substrate through physical contamination, without requiring viral replication or completion of a developmental cycle within the vector. Accordingly, the detection of viral RNA in recipient media should be interpreted as evidence of mechanical transfer of viral genetic material rather than biological transmission. In contrast, biological vector competence requires successful infection of the vector, viral replication within internal tissues, dissemination beyond the midgut barrier, invasion of the salivary glands, and subsequent transmission during feeding, none of which were evaluated in the present study (19,20).

Discrepancy between Serological and Molecular Detection

The present study revealed a marked discrepancy between the serological and molecular findings. Although all fifty blood samples tested positive for anti-Coxsackievirus IgM using the rapid antibody assay, viral RNA was detected by RT-qPCR in only 22 stool samples (44%). This apparent inconsistency does not represent a contradiction between the two diagnostic approaches, but rather reflects the fundamentally different biological targets that they measure. Serological assays detect the host immune response, which may persist after the decline of active viral shedding, whereas RT-qPCR directly detects viral RNA and therefore depends on the presence of sufficient viral genetic material in the specimen at the time of collection. Consequently, molecular positivity is influenced by several factors, including the stage of infection, specimen type, timing of sample collection relative to viral shedding, RNA extraction efficiency, and the presence of amplification inhibitors, all of which may reduce assay sensitivity and contribute to false-negative molecular results (21,22,23).

Current recommendations for enterovirus surveillance emphasize that molecular detection and genotyping should

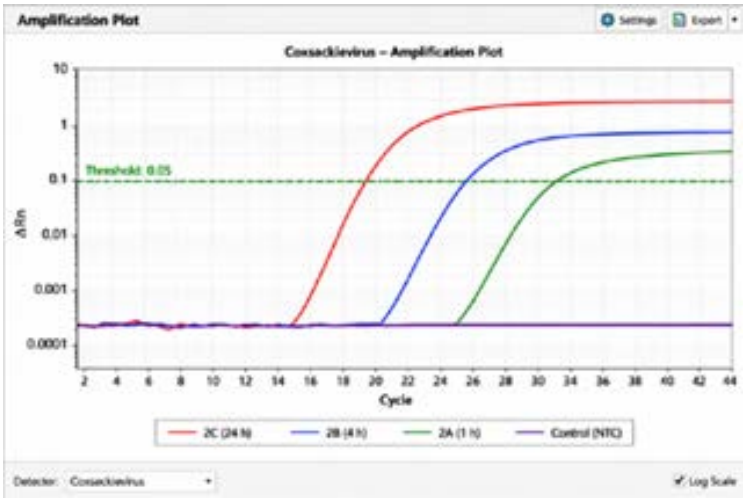

Figure 4. Amplification plots of Coxsackievirus after mechanical transmission by female Culex molestus mosquitoes at different time intervals; from which molecular evidence is derived of the regularity of the sigmoidal amplification curves of the three positive samples, with the negative control remaining stable below the reference threshold line

Table 8. Comparison of Coxsackievirus Molecular Detection Results between Original and Mechanically Transported Samples at Different Exposure Periods

Parameter	Original Samples (Coxsackievirus)	Mechanical Transmission (1 hour)	Mechanical Transmission (4 hours)	Mechanical Transmission (24 hours)
No. of samples	22	1	1	1
Mean Ct ± SD	28.75 ± 5.31	36.92 ± 0.44	33.61 ± 0.38	29.84 ± 0.31
Viral load (copies/µL)	≈ 5.0×10^3	4.5×10^0	4.1×10^1	5.4×10^2
RT-qPCR result	Positive	Positive	Positive	Positive
ΔCt from original	—	+8.17	+4.86	+1.09
Viral load relative to the original (%)	100%	0.09%	0.82%	10.8%
Evidence of transmission	—	Confirmed transmission	Confirmed transmission	Confirmed transmission
Correlation coefficient (r)	—	-0.934	-0.934	-0.934
Coefficient of determination (R^2)	—	0.872	0.872	0.872
P-value	—	<0.001	<0.001	<0.001

Table 9. Coxsackievirus RNA Recovery Rate after Mechanical Transfer

Exposure Time	ΔCt from the Original Source	Recovery Rate (%)
1 hour	+ 8.17	0.09%
4 hours	+ 4.86	0.82%
24 hours	+ 1.09	10.8%

Table 10. ANOVA Analysis of the Effect of Exposure Duration on Ct Values

Source of Variation	F-value	*P*-value	Significance
Exposure time	96.3	<0.001	Highly significant

be prioritized for laboratory confirmation because they provide direct evidence of viral circulation and enable accurate characterization of circulating strains. However, molecular findings should always be interpreted in conjunction with specimen type, sampling time, and the patient's clinical presentation (21, 24). Therefore, the lower molecular positivity rate observed in the present study should not be regarded as conflicting evidence with the serological results, but rather as a reflection of the distinct diagnostic windows and biological significance of the two methods. From a methodological perspective, these findings demonstrate that selecting source material for mechanical transfer experiments based solely on serological positivity may lead to the inclusion of specimens lacking detectable viral RNA. Accordingly, the use of RT-qPCR-confirmed positive specimens as the contamination source represents a major strength of the present experimental design, ensuring that the transmission assay was performed using virologically confirmed material rather than relying exclusively on serological evidence.

Significance of Positive Receptor Media and Negative Controls

One of the most important findings of the present study was the detection of Coxsackievirus RNA in all previously virus-negative receptor media following exposure to mosquitoes that had contacted the positive source, whereas no amplification was observed in any of the negative controls. In the Coxsackievirus assay, the positive control produced the expected amplification (Ct = 25.1), while both the negative extraction control and the no-template control (NTC) remained consistently negative (Table 12). The inclusion of these controls is fundamental to ensuring the validity of RT-qPCR results and is consistent with the recommendations of the Minimum Information for

Table 11. Relationship between Exposure Duration and Molecular Detection Results

Virus	Correlation Coefficient (r)	Coefficient of Determination (R^2)	*P*-value	Interpretation
Coxsackievirus	-0.934	0.872	<0.001	Strong negative correlation between exposure time and Ct value

Table 12. Laboratory Control Results for the Coxsackie Virus RT-qPCR Assay

Assay	Positive Control (Ct)	Negative Extraction Control	NTC (No-Template Control)	Internal Control	Run Validity
Coxsackievirus 5′-UTR RT-qPCR	25.1	No Ct	No Ct	Acceptable	Successful

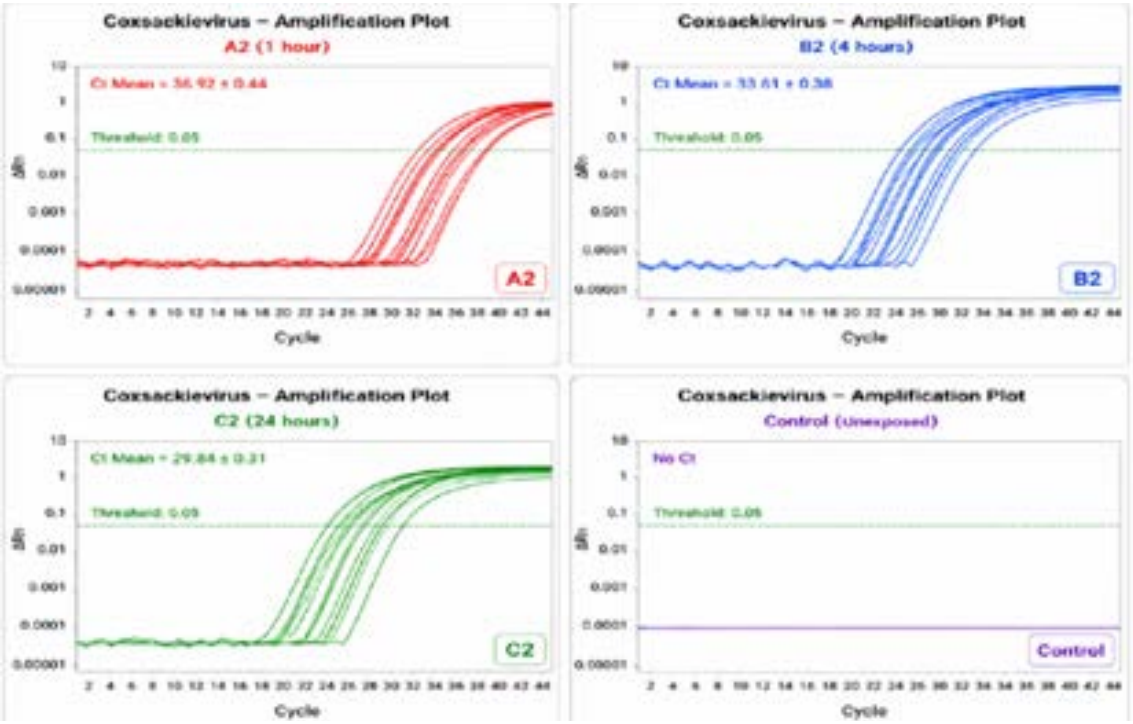

Figure 5. Comparison of amplification curves for Coxsackie virus in mechanically transmitted samples by female *Culex molestus* mosquitoes at different time intervals. A systematic sequence of decreasing cycle threshold values is observed, coinciding with the widening of the time window for insect contact

Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, which emphasize the use of appropriate positive and negative controls, assessment of extraction quality, detection of PCR inhibition, and transparent reporting to ensure analytical reliability and reproducibility (25, 26).

The strength of the present findings lies not simply in the detection of viral RNA after mosquito exposure, but in the logical sequence of experimental evidence: a molecularly confirmed positive source, a previously negative receptor medium, mosquito-mediated contact between the two, and subsequent detection of viral RNA only in the exposed receptor media while all molecular controls remained negative. This experimental design substantially reduces the likelihood that the observed RT-qPCR positivity resulted from laboratory contamination or analytical artifacts. Nevertheless, these findings should be interpreted cautiously. Detection of viral RNA following mosquito-mediated transfer demonstrates the movement of viral genetic material but does not provide evidence that the transferred virus remained infectious or capable of replication. Therefore, the present findings should be interpreted as evidence of mechanical transfer of Coxsackievirus RNA rather than proof of biological mosquito-borne transmission. Similar conclusions were reported by Balaraman et al. (18), who demonstrated that house flies (Musca domestica) mechanically transferred detectable SARS-CoV-2 RNA after exposure to contaminated substrates under laboratory conditions. Importantly, the authors concluded that RT-qPCR positivity indicated transfer of viral RNA rather than biological vector competence or confirmed infectivity. Likewise, the MIQE guidelines emphasize that reliable molecular detection should not be interpreted as evidence of biological infectivity in the absence of complementary infectivity assays (25).

Variation in Viral Load of Original Samples

The Ct values of the original RT-qPCR-positive samples ranged from 19.2 to 37.6 (mean 28.75 ± 5.31), indicating substantial variation in the amount of Coxsackievirus RNA among the clinical specimens. Furthermore, 45.4% of the positive samples were classified as having a low viral load, whereas the remaining samples were distributed between moderate and high viral load categories (Table 5). Such variability is consistent with the dynamic nature of enterovirus shedding, which is influenced by multiple biological factors, including the stage of infection, timing

of specimen collection, viral genotype, host immune response, and individual host characteristics (21, 27). The observed variation in viral RNA concentration is biologically meaningful because it directly affects the amount of viral genetic material available for acquisition during mosquito contact. Accordingly, sources containing higher concentrations of viral RNA would be expected to make larger quantities of viral material available for acquisition and subsequent transfer, although the relationship between the viral load of the source and the quantity of transferred RNA was not formally quantified in the present study. These findings suggest that the efficiency of mechanical transfer is influenced not only by the duration of mosquito–source contact but also by the level of viral contamination in the original source. From an ecological perspective, these findings further suggest that environments containing highly contaminated fecal material may provide greater opportunities for the mechanical acquisition and subsequent transfer of viral genetic material than environments with lower levels of contamination. However, these findings should be interpreted as evidence of RNA transfer rather than biological transmission, since the present study did not assess viral infectivity or replication following transfer (20).

Effect of Exposure Duration: Time-Dependent Physical Accumulation

One of the most interesting findings of the present study was the decrease in Ct values as mosquitoes remained exposed for longer periods to the contaminated source. Notably, Ct values decreased by more than seven cycles between the 1-h and 24-h exposure groups, accompanied by an increase in the estimated viral RNA quantity, supporting a time-dependent physical accumulation interpretation. Longer exposure periods may increase the probability and frequency of mosquito interactions with contaminated substrates, including landing, walking, surface contact, and feeding, thereby increasing opportunities for acquiring viral material. This concept is consistent with the principles of mechanical transmission, where the amount of transferred pathogen material depends on the level of source contamination, contact frequency, and the interval between acquisition and subsequent deposition, rather than requiring pathogen replication within the insect (28). The present findings further suggest that exposure duration influenced not only the probability of detecting viral RNA but also the quantity of recovered genetic material. However, the current study cannot determine whether the increased RNA recovery resulted from accumulation on specific external body structures, such as legs, hairs, or mouthparts, or from a combination of multiple contact pathways. Therefore, the possibility of external attachment should be considered as a proposed explanatory mechanism rather than a confirmed pathway. Since this study evaluated viral RNA detection only, the results do not demonstrate viral replication or biological transmission by Culex molestus, but rather indicate the potential role of mosquitoes in the mechanical carriage and transfer of viral genetic material under experimental conditions (29).

Demonstrating the Mechanical Transfer of Viral Genetic Material

The most important result from our study is that we found a positive amplification of Coxsackievirus RNA in all samples taken after contact with the positive viral source and exposed to mosquitoes, whereas all control samples remained negative and showed no amplification. This combination of three experimental components (positive source, positive receiver media after insect mediation, and negative unexposed control) provides strong experimental evidence for the physical transfer of viral genetic material under controlled laboratory conditions. This interpretation is further supported by the successful molecular controls, with the positive Coxsackievirus control amplifying at Ct = 25.1, while both the negative extraction control and the no-template control (NTC) showed no amplification, excluding detectable contamination during the analytical workflow (20). Mechanical transmission does not require pathogen replication or development within the vector but instead depends on the physical acquisition and subsequent transfer of infectious material or viral genetic material from one substrate to another (30). Therefore, the findings of the present study are conceptually distinct from studies investigating biological mosquito transmission, which require evidence of viral infection of mosquito tissues, dissemination beyond the midgut, and invasion of the salivary glands before transmission can occur (20). It is important to emphasize that the present study demonstrates the mechanical transfer of Coxsackievirus RNA only; it neither investigated viral replication within Culex molestus nor demonstrated that the transferred material contained infectious viral particles capable of initiating subsequent infection. This distinction is essential because RT-qPCR detects viral RNA with high analytical sensitivity but does not determine viral viability or infectivity (30).

Effect of Exposure Duration on Mechanical Transport Efficiency

Another important finding of the present study was the clear temporal trend observed in the mechanical transport process. The Ct value decreased from 36.92 after 1 h of exposure to 33.61 after 4 h, and further declined to 29.84 after 24 h. Concurrently, the calculated viral load increased from 4.5×10^0 to 4.1×10^1 and finally to 5.4×10^2 copies/µL, suggesting that, under the conditions of the present experiment, mechanical transport was not simply an instantaneous or binary event but rather a quantitative process influenced by exposure duration (31). Prolonged exposure of mosquitoes to a virus-contaminated source is expected to increase the frequency of landings, feeding attempts, and physical contacts, thereby increasing the probability of acquiring viral RNA and subsequently depositing it into the receiving medium (20). Statistically, the reduction in Ct between the 1-h and 24-h exposure groups exceeded seven amplification cycles and was

accompanied by a marked increase in the estimated viral load, supporting the hypothesis proposed in the present study that viral RNA accumulation on mosquito external structures may occur in a time-dependent manner. However, this interpretation should be considered a post hoc explanation based on the quantitative trend observed in the experimental data rather than direct evidence of viral localization on specific mosquito body parts, since separate analyses of the legs, mouthparts, or other external structures were not performed. Future studies employing anatomical dissection, serial surface smears, scanning electron microscopy, or immunofluorescence techniques would provide valuable evidence for identifying the anatomical sites involved in viral RNA retention and its subsequent loss (20).

Statistical Relationship between Time and the Amount of Transferred RNA

Statistical analysis confirmed the experimental trend observed in the present study. Pearson's correlation analysis demonstrated a strong negative association between exposure duration and Ct values ($r = -0.934$, $R^2 = 0.872$, $P < 0.001$), while one-way ANOVA revealed a statistically significant difference among exposure durations ($F = 96.3$). The inverse relationship between exposure time and Ct values is consistent with an increased opportunity for mosquitoes to acquire viral RNA following prolonged contact with the contaminated source, resulting in greater quantities of detectable viral RNA in the receiving medium (31). The strength of this correlation supports a clear temporal association under the experimental conditions. Nevertheless, it is important to emphasize that an R^2 value of 0.872 should not be interpreted as evidence that exposure time alone explains 87.2% of the biological mechanism underlying viral RNA transport. Rather, the coefficient of determination describes the proportion of variance explained by the fitted statistical model and does not establish a cause-and-effect relationship (32). In the present study, causal inference is instead supported by the controlled experimental design, predefined exposure periods, appropriate negative controls, and the consistent association between Ct values and the estimated viral RNA load.

Molecular Recovery Efficiency after Transfer

Comparing the original source material with the recovered material after mechanical transfer, the recovery rate was 0.09% after 1 h of exposure, increasing to 0.82% after 4 h and reaching 10.8% after 24 h. Concurrently, the ΔCt difference between the original source and the recovered material decreased from +8.17 to +4.86 and finally to +1.09 cycles. One of the most important findings of the present study is that increasing mosquito contact time not only enhanced the probability of qualitative viral RNA detection but also increased the proportional amount of viral genetic material recovered after transfer. The ΔCt value after 24 h (29.84 ± 0.31) approached that of the original positive samples (28.75 ± 5.31), suggesting greater molecular recovery with prolonged exposure. However, the term "recovery rate" is used here strictly as a molecular estimate derived from RT-qPCR data and should not be interpreted as the proportion of infectious viral particles transferred. Likewise, the increase in estimated copy number and the reduction in Ct values indicate a greater amount of amplifiable viral RNA but do not demonstrate the persistence of viral infectivity, since RT-qPCR detects viral nucleic acid rather than viral viability or infectivity (31).

The Environmental Significance of Culex molestus Selection

Given the ecological characteristics of Culex molestus, the mosquito species used in the present study, these findings have important environmental implications. Culex molestus is widely recognized as a mosquito closely associated with urban habitats, particularly enclosed and semi-enclosed environments such as underground structures, sewage systems, and organically enriched aquatic habitats. Its adaptation to anthropogenic environments makes it an ecologically relevant species for investigating environmentally mediated pathogen transport (33). Recent population genomic studies have substantially revised the traditional hypothesis that Culex molestus originated only within modern underground transport systems. Instead, current evidence indicates that this ecotype likely adapted to enclosed human-associated environments more than one thousand years ago, long before the emergence of modern urban infrastructure (34). These findings reinforce the importance of studying the interactions between this ecological niche and contaminants associated with human activities rather than limiting its significance solely to biological vector competence. Therefore, the widespread occurrence of Culex molestus in urban environments characterized by poor wastewater and waste management supports the need for future field investigations to evaluate the hypothesis of mechanical transport of enteric viral RNA under natural environmental conditions. Nevertheless, extrapolation from laboratory observations to epidemiological significance requires carefully designed field studies demonstrating that the same sequence of environmental contacts occurs in nature (33, 34).

Novelty and Scientific Originality of the Study

The novelty of the present study lies in its departure from the conventional approach of investigating mosquitoes as biological vectors of Coxsackievirus. Instead, it evaluated a distinct experimental model based on the mechanical transfer of viral genetic material from a positive fecal source to a previously negative fecal medium while incorporating exposure duration as a quantitative experimental variable. The originality of this work is further strengthened by combining an urban mosquito model (Culex molestus), an enterovirus that is not traditionally regarded as mosquito-borne, a multi-time-point experimental design, and quantitative molecular assessment using Ct values, estimated viral load, ΔCt, recovery rate, and independent

molecular controls.

Integrated Interpretation of Study Results

In light of the combined findings, the present study proposes an interpretative model consisting of a coherent sequence of events: a positive viral source, mosquito contact with the contaminated source, physical acquisition of viral material, transient retention influenced by contact conditions, transfer of the mosquito to a clean medium, redeposition of a portion of the acquired material, and subsequent detection of viral RNA by RT-qPCR. This model is supported by five consistent observations: successful detection of viral RNA under all exposure conditions, consistently negative control samples, progressive reduction in Ct values with increasing exposure time, a corresponding increase in the estimated viral load, and an increase in the calculated recovery rate from 0.09% to 10.8%. Collectively, these findings provide stronger evidence than the simple detection of viral RNA in environmental mosquitoes because the present experimental design demonstrates the directed transfer of viral genetic material from a confirmed positive source to a defined recipient under controlled laboratory conditions. However, the findings should not be interpreted as evidence that Coxsackievirus is a mosquito-borne virus, since the present study did not investigate viral replication, dissemination, or transmission of infectious viral particles within the mosquito. Rather, the results support the hypothesis that mosquitoes may contribute to the passive environmental movement of viral genetic material under suitable conditions, representing a concept that is distinct from classical biological vector competence (20, 31).

Comparison with the Concept of Mechanical Transmission in Other Insects

Historically, most published studies investigating the mechanical transmission of enteric pathogens have focused on houseflies and other synanthropic insects that frequently move between fecal material, organic waste, and human environments, rather than mosquitoes (35). These studies have demonstrated that insects inhabiting contaminated environments may mechanically transport pathogens or their genetic material to new surfaces or substrates through repeated physical contact (35). The significance of the present study lies in shifting this concept toward an urban mosquito species, Culex molestus, which is closely associated with organically enriched aquatic habitats. This does not imply that mosquitoes possess the same mechanical transmission efficiency as houseflies; rather, the present experiment specifically examined whether mosquitoes could transfer detectable viral RNA immediately after contact with a virus-rich source to a previously uncontaminated recipient medium. The successful molecular detection observed in the present study supports the feasibility of this process at the RNA level and highlights an underexplored area of research concerning the environmental movement of enteric viral genetic material by mosquitoes. However, these findings should not be interpreted as evidence of biological vector competence or mosquito-borne transmission of Coxsackievirus (20).

Study Limitations and Future Research Prospects

Despite the rigorous experimental design and appropriate laboratory controls, the present study has several important limitations. First, RT-qPCR detects viral RNA with high analytical sensitivity but does not distinguish between infectious and non-infectious viral particles (31). Second, although viral RNA was successfully detected following mosquito-mediated transfer, the anatomical sites responsible for retention of viral material on the mosquito body were not investigated. Third, the laboratory conditions employed in this study cannot fully reproduce the environmental complexity encountered under natural field conditions. Finally, although the present findings demonstrate mechanical transfer of viral RNA, they do not determine the duration for which infectious virus, rather than viral RNA alone, can persist on the mosquito body. Consequently, future investigations should include infectivity assays using appropriate cell culture systems, comparative analyses of whole mosquitoes and individual body parts (including the legs, mouthparts, and digestive tract), time-course experiments evaluating the persistence of viral material following removal from the source, assessment of different transmission distances, and subsequent validation under semi-field and field conditions (20,33).

*Interpretive Conclusion of the Discussio*n

When combining the results of the current study with current knowledge of enterovirus ecology and the principles of mechanical transmission, the following conceptual model can be proposed. Mosquitoes are exposed to a source containing a detectable viral load. The probability of acquiring viral material increases with repeated contact, after which a proportion of the acquired material is temporarily retained and transferred to a previously uncontaminated substrate. The amount of recovered viral RNA increases with prolonged exposure, although the transfer process remains associated with quantitative loss relative to the original source. The principal contribution of the present study is the demonstration that the molecular mechanical transfer of Coxsackievirus RNA by Culex molestus follows both time-dependent and source-dependent patterns rather than representing a purely random physical event. Whereas previous studies have established the theoretical basis of mechanical transmission and enterovirus persistence, the present work provides an experimental framework integrating these concepts into a single quantitative model. The principal limitation of the present study is that viral infectivity was not assessed; therefore, the most appropriate conclusion is that Coxsackievirus RNA, rather than infectious virus, was mechanically transferred under controlled laboratory conditions, with the amount of recovered viral RNA increasing as exposure duration increased (36). Future studies should determine whether the transferred virus

remains infectious by using cell culture or infectivity assays, identify the anatomical sites of viral retention on the mosquito body, determine the duration of infectious virus persistence, and evaluate this phenomenon under semi-field and field conditions (37-41).

Conclusion

1. Demonstration of Non-Traditional Mechanical Transmission: The study demonstrated in vitro the ability of female *Culex molestus* mosquitoes to act as mechanical and physical vectors for Coxsackievirus RNA from contaminated fecal sources. This opens new research avenues regarding the role of urban mosquitoes in the dynamics of enteroviruses not traditionally classified as arboviruses.
2. Time-Dependent Kinetics of Transmission: The study showed that molecular mechanical transmission is not a random or abstract qualitative event, but rather a gradual, time-controlled quantitative process. The efficiency of molecular recovery and the viral load transferred are directly proportional to the duration of the insect's contact with the contaminated source. This was further supported by the very strong inverse statistical correlation between exposure time and Ct values.
3. Multifactorial Efficiency: The efficiency of molecular mechanical transmission is affected by the interaction of two crucial factors: the initial viral load at the source (which the study showed to be heterogeneous in clinical samples) and the duration of insect exposure. This means that the risk of mechanical transmission increases sharply in environments with high viral contamination and continuous contact.
4. Limitations of Serological Diagnosis in Transmission Studies: The clear discrepancy between complete seropositivity (100% for IgM) and the low percentage of genome-bound feces (44% by RT-qPCR) demonstrates the limitations of relying solely on serological testing for selecting viral sources for experimental transmission studies. This necessitates molecular confirmation to ensure accurate modeling.
5. Reliability and Rigor of the Experimental Model: The absolute negativity of the control groups and the extraction and amplification (NTC) controls reinforced the reliability of the experimental design, confirming that the recovery of viral RNA in the recipient media was an exclusive result of the experimental mosquito-borne transmission pathway and ruling out any possibility of laboratory contamination.
6. Scientific Limitations and Future Prospects: The most accurate scientific conclusion of the study is that it demonstrates the "mechanical molecular transfer" of genetic material, without definitively proving the ability to cause infection or biological transmission. This is because RT-qPCR does not measure viral viability, thus establishing a much-needed experimental basis for employing cell culture in future studies.

Authors' Contribution

Conceptualization: Rusul Abdulnabi Wali, Saif Jabbar Yasir
Data curation: Rusul Abdulnabi Wali
Formal analysis: Rusul Abdulnabi Wali, Rafid Abbas Ali
Investigation: Rusul Abdulnabi Wali
Methodology: Rusul Abdulnabi Wali, Saif Jabbar Yasir
Project administration: Rafid Abbas Ali
Resources: Saif Jabbar Yasir, Rafid Abbas Ali
Software: Rusul Abdulnabi Wali
Supervision: Rafid Abbas Ali, Saif Jabbar Yasir
Validation: Saif Jabbar Yasir
Visualization: Rusul Abdulnabi Wali
Writing–original draft: Rusul Abdulnabi Wali
Writing–review & editing: Rafid Abbas Ali, Saif Jabbar Yasir

Conflicts of Interests

The authors declare that they have no conflicts of interest.

Data Availability Statement

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

All procedures involving human participants were reviewed and approved by the Research Committee of the Training and Human Development Center, Thi-Qar Health Directorate, Iraqi Ministry of Health, Nasiriyah, Iraq (Approval No.: 4098; Research Committee decision No.: 25/2026; Date: January 2026). The study was performed in accordance with the ethical standards of the responsible institutional committee and with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all participants prior to the collection of blood and stool samples.

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