



Isolation and molecular detection of Marek's disease virus from layers chicken farms in Iraq

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Abstract

Marek's disease (MDV) is an essential oncogenic and immunosuppressive disease of chickens, distributed worldwide, mainly affecting all birds at different ages, leading to significant economic losses caused by the Gallid herpesvirus of Alphaherpesvirinae. This study involved the isolation of Marek's Disease virus-Gallid herpesvirus2, from three clinical specimens (liver, spleen, and feather follicles) collected from layer flocks suspected with MDV for the first time in Iraq. Clinical samples showed hyperplasia, necrosis foci, and hemorrhage on their surface, significant lesions of feather follicle enlargement, and detection of tumor masses occasionally. Chicken embryo fibroblast (CEF) cell cultures were used for virus isolation; prepared samples were inoculated on CEF for two passages. The main cytopathic effects were cell rounding, cell clusters, Syncytia forming, and plaque formation starting 5 days post-inoculation. The isolates were confirmed by a Taqman real-time PCR technique with a specific primer against viral pp38. Additionally, the pathogenesis of isolates and clinical samples was investigated through molecular technique by detecting Marek's virus genome's tandem 132 repeat region. All tested samples give a single amplification band, indicating that the current circulating strains under investigation are of high virulence. This study will support the need for a better understanding of Marek's virus disease in Iraqi poultry farms and enhance its diagnosis to restrict its passive impact on the poultry sector in the country. In summary, this study aimed to isolate Marek's Disease virus from infected layers' flocks on cell culture and detect the virulence of these field strains through molecular techniques.

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Introduction

Marek's disease is an infectious, highly contagious viral disease of birds, primarily chickens, characterized by its high oncogenicity and immunosuppression in affected flocks, leading to high economic losses with its distribution worldwide (1). The disease was first described in 1907 by Josef Marek. According to its clinical manifestation, the virus was not identified until 1967, when it was isolated and characterized as herpesvirus; soon after that, a vaccine was designed to mimic the effect of the virus on poultry flocks. Over the years, with continuous administration and

development of vaccines, the virus showed an increased virulence rate that needs more potent vaccine and management (2).

The disease is caused by an alphaherpesvirus known as Gallid herpesvirus, with three serotypes termed Gallid herpesvirus 2,3 and Mellagrid herpesvirus HVT (3), carry a large DNA genome of about 180 Kbp that encodes for more than 200 genes, among them some of the unique virulence genes like meq, vil-8, pp38, pp24, vlip (4,5). Initially, the virus was diagnosed through isolation on cell culture, which was regarded as the gold standard; later, with the appearance of molecular techniques, PCR became the most desirable test

for diagnosis due to its low costs and high accuracy as well as providing a reliable tool to study different genes and virulence factors (6).

In Iraq, the poultry industry is suffering from multiple pathogenic challenges, such as Avian influenza (7,8), Newcastle Disease (9), Infectious Bronchitis (10,11), Sporadic infection with Infectious Laryngotracheitis (12), and recently increased cases of Marek's Disease (13).

Materials and methods

Ethical approval

All experimental animals were used in this study approved by the Scientific Committee of the College of Veterinary Medicine, University of Baghdad in compliance with the ethical principles' guidelines on the care and use of animals in research (approval no: 1240 P.G, date 27/6/2024) of animal welfare.

Samples collection and necropsy examination

In this study, 50 layer farms suspected of Marek's Disease virus infection from different Iraqi provinces (Baghdad, Wasit, Diyala, Kirkuk, Al Najaf, Karbala, Al Anbar, Al Basrah, Al Diwaniya, and Thi-Qar) along two years (2022-2023) were investigated. A post-mortem examination was done on chickens after euthanizing them. One hundred sixty samples were collected from visually suspected organs (liver, spleen, kidney, feather follicles, and tumor mass). Those organs were brought to the Virology laboratory/ Central Veterinary laboratories, chopped and minced, and stored at -70°C until the test.

Marek's Disease virus isolation by cell culture

The experimental isolation of Marek's Disease virus on cell culture involved the preparation of chicken embryo fibroblast (CEF) cell culture from 10-day-old chick embryos. Briefly, chick embryos were euthanized, and head and terminates were removed, chopped, and washed with sterile

phosphate buffer saline PBS. The tissue pieces were digested with 0.25% Trypsin in a magnetic stirrer water bath for about 10 minutes at 37°C. The suspension was filtered into a sterile beaker topped with a triple gauze layer, and growth cell media supplied with 10% fetal bovine serum was added to the filtrate, then divided into tubes, and centrifuged at 1500 rpm for 10 minutes at 4°C. The pelleted cells were resuspended with a growth medium, poured into a 25cm² growth culture flask, and incubated at 37°C till 80% of mono-fluctuant cell growth was reached. Marek's virus was inoculated on cell culture at 0.25ml after filtration on a 0.45ul millipore filter to avoid contamination. Inoculated cell culture flasks were observed daily and compared with control culture flasks. The isolation process followed the recommendations of (14).

DNA extraction

Viral DNA was purified from the suspected cell culture by detaining these cells from the culture flask surface through multiple freezing and thawing processes. Then the cell was suspended into a centrifuge tube and centrifuged for 10 minutes at 2000 rpm under 4°C, then 200ul of the pelleted cells were collected for purification of viral DNA using a QIAamp DNA kit from Qiagen® according to the manufacturer's instructions. The purified DNA was kept under -70°C till the test.

Confirming Marek's virus isolation on cell culture

To detect the viral DNA of Marek's Disease virus, three samples were collected from inoculated cell culture; two of them were from two cell culture flasks after pelleted their cells by centrifugation at 2000 rpm for 5 minutes, while the third one was collected from the supernatant of centrifuged cell culture. In addition to positive and negative controls, these three samples were tested with Taqman real-time PCR technique using a primer-probe set that targets Marek's Disease virus pp38 protein (Table 1).

Table 1: Primer-Probe set used for the detection of Marek's Disease virus by a Taqman real-time PCR technique

Oligo Name	Sequence 5'-3'	Reference
MDV PP38F	GAG CTA ACC GGA GAG GGA GA	(15)
MDV PP38R	CGC ATA CCG ACT TTC GTC AA	
MDVPP38 Pr	FAM-CTC CCA CTG TGA CAG CC-BHQ1	

These primers were manufactured by ALPHA DNA / Canada and mixed with the reaction buffer Go Taq® Probe 1-Step RT-qPCR system manufactured by Promega. For each sample, the reaction mix was prepared with 2x buffer solution mixed with 0.4ul of enzyme mix, 1ul of 10 pmol forward and reverse primers, 0.5ul of Taqman probe, 0.05ul of Rox passive reference dye, and 2.05ul of nuclease-free water. The reaction buffer mix was finalized to 20 ul by

adding 5ul of viral DNA template. This mix was passed under a specific thermal condition of initial denaturation and hot start activation at 95°C for 2 minutes, followed by 40 cycles of 95°C for denaturation and 60°C for annealing and extension elapsed for 3 and 30 seconds, respectively. The fluorescence signal was read during the extension step using the FAM filter, and the results analysis was carried out according to instrument settings. The amplification process

took place using an Applied Biosystems 7500 fast thermal cycler.

Study of viral virulence by Conventional PCR

In order to study the pathogenicity of Marek’s disease virus isolates, a conventional PCR experiment with a primer set that targets the tandem repeat 132 bp region was selected (Table 2). This gene was amplified using the GoTaqG2 Green Master Mix from Promega with a final volume of 25ul composed of 12.5ul of 2X master mix, 1ul of both forward and reverse primers 5.5ul of nuclease-free water, and 5ul of

DNA template. The thermal condition for this reaction is started with 4 minutes at 95°C for initial denaturation, followed by 45 cycles of 1 minute at 95°C, 1 minute at 62°C for annealing, 2 minutes at 72°C for extension, then one step at 72°C for 10 minutes for final extension. The thermal reaction was done using the Applied Biosystem ProFlex PCR system. The results were visualized on 1.5% agarose stained with ethidium bromide, electrophoresis with 70 Volts for 90 minutes, and amplicons were visualized on a UV light source.

Table 2: primers set used to study the pathogenicity of Marek's Disease virus isolates by conventional PCR

Oligo name	Oligo sequence	Amplicon	Reference
132bp Repeat.F	5'- ATG CGA TGA AAG TGC TAT GGA G-3'	variable	(2)
132bp Repeat.R	5'- ATC CCT ATG AGA AAG CGC TTG A-3'		

Results

Ethical approval

Ethical approval was granted through the local committee of animal care and use at the College of Veterinary Medicine within the University of Baghdad (Number 1240 on 2024/06/27).

Gross lesions

At necropsy, gross changes were observed in visceral organs, including hyperplasia of the spleen with apparent necrotic foci on its surface (Figure 1). Kidneys were also found pale in color, losing their lobulated appearance (Figure 2). The liver occupies most of the abdominal cavity, with multiple necrotic foci on its surface and red dots representing petechial hemorrhage (Figure 3).



Figure 2: Kidneys infected with MDV.

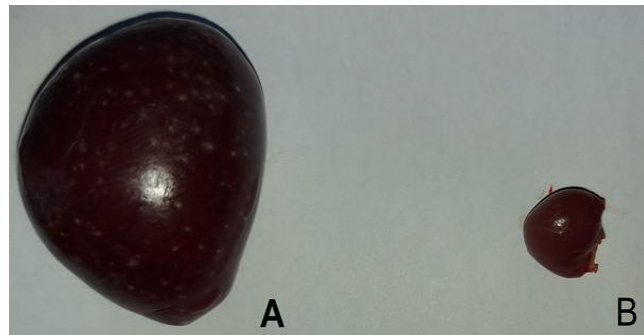


Figure 1: (A) hyperplastic spleen appears with multiple necrotic foci on its surface compared with (B) normal size spleen from non-infected chicken.

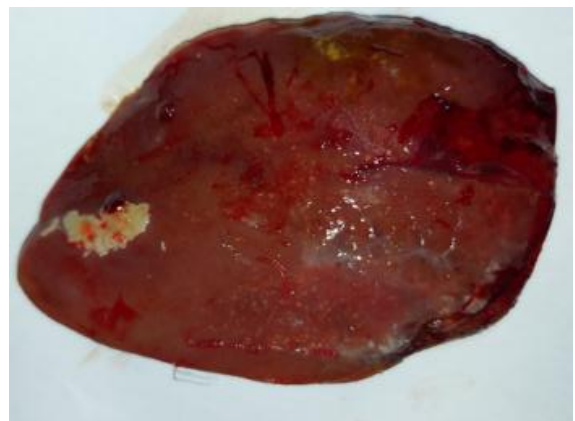


Figure 3: part of the liver infected with MDV show enlargement, paleness, and loss of lobulation with multiple foci of necrosis on its surface.

A very significant and characteristic lesion was found in feather follicles that appeared very enlarged and pale in color (Figure 4). Multiple irregular masses were also observed within the abdominal cavity (Figure 5).



Figure 4: Enlarged feather follicles embedded in the skin adipose tissue represent the main site for virus-free replication and the main route for disease transmission.

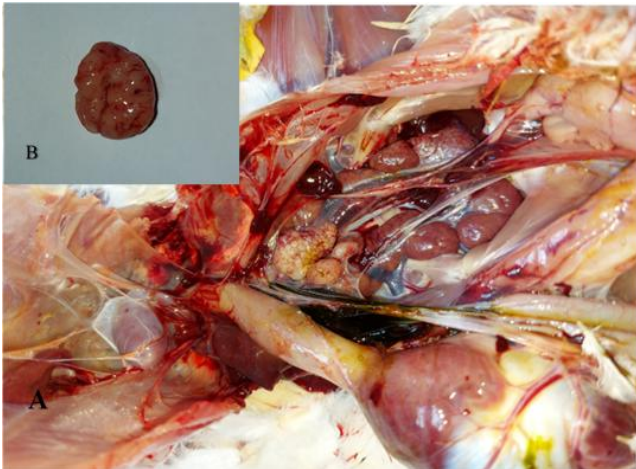


Figure 5:(A) multiple tumor masses are seen within the carcass. (B) isolated Tumor mass.

Virus isolation on cell culture

After inoculating the CEF cell culture with a Marek's Disease virus sample, a continuous daily check was followed. The initial cellular cytopathic effect (CPE) was first identified on the 5th day post-inoculation when the infected culture cells started to round and form clusters; giant cells (Syncytia) were also observed, and plaques started to form within cultured cells until the 8th day of inoculation. A control cell culture was also maintained and used to compare with the infected culture flask (Figure 6).

Marek's virus detection by real-time PCR

The amplification process of extracted DNA from cell culture revealed the presence of three amplification blots (Figure 7). One of them was the reaction control, which gives

a Ct value of 24.8, while the other two amplification curves were for the tested samples S1 and S2, which showed Ct values of 33 and 36, respectively. Finally, a very late and short amplification curve of S3 gives a Ct value above 38.5 and was ignored.

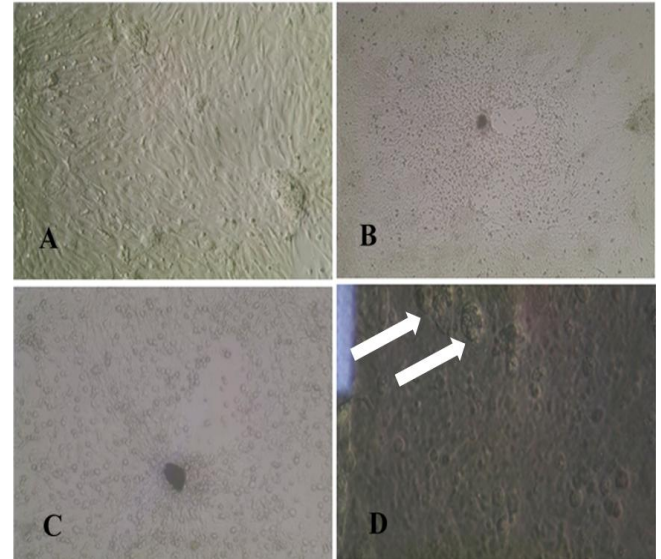


Figure 6: Marek's Disease virus isolation on CEF (A) Control cells (B) Cytopathic effect of the virus with cell rounding, Clustering, and plaque formation. (C) higher focus on CPE (D) formation of Syncytia due to virus effects.

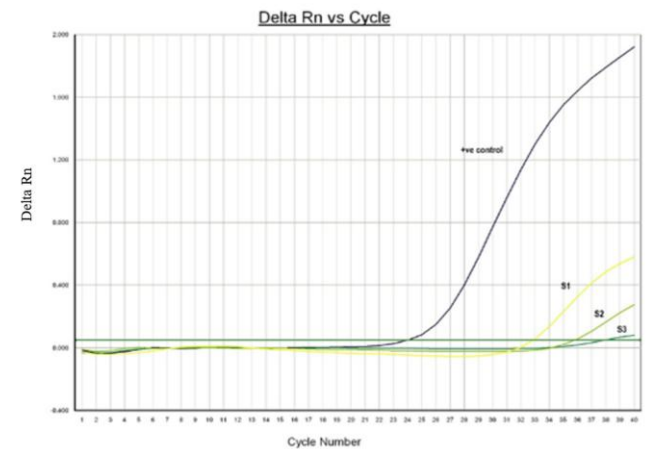


Figure 7: Amplification blot of real-time PCR with Control, S1 and S2 samples from pelleted cells, S3 sample from supernatant

Conventional PCR to study viral virulence

The thermal amplification of Marek's virus samples for detecting the tandem repeats 132 bp genes revealed at results visualization on agarose, a single amplicon band that weighed around 320 bp (Figure 8).

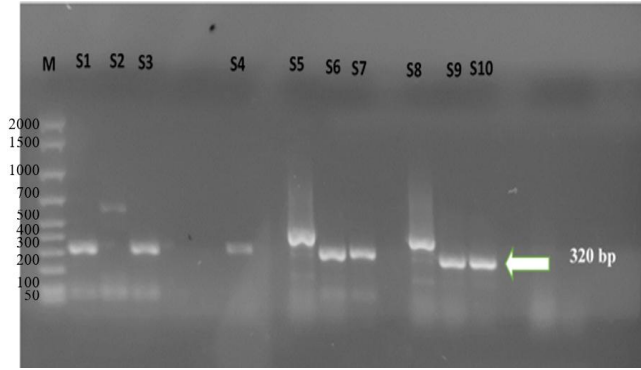


Figure 8: electrophoresis of PCR product of 132 tandem repeat region, amplicon size is about 320 bp with some samples showed higher product weight, a single band reflects the virulent nature of the virus, while multiple band for any sample reflect that this virus was attenuated and lost its virulence. 2% Agarose, Ladder, Biorad 50-2000bp.

Discussion

Splenomegaly, hepatomegaly, and splenomegaly represent a standard, non-specific pathological change for several avian diseases, including MDV-infected birds, as the spleen represents one of the main lymphoid organs at which the virus spends a part of its infectious cycle. At the same time, the liver and kidneys are very vital organs. Their enlargement occurs due to the attraction and continuous proliferation of transformed lymphoma cells, especially T-cells, in these organs, and finally, hemorrhage, necrosis, and transplantation are seen within; these observations are matched with case description (16,17).

The macroscopic appearance of feather follicles represents a significant indicator for MDV infection, which agrees with the (18) description, these tissues are the main site for infective viral replication as well as, these tissues are the lonely natural carriers for viral transmission, and their enlargement is the natural outcome for attracting inflammatory cells especially the latently infected T lymphocytes converting these tissues into a huge viral reservoir for the virus infectious cycle and appearing as nodular skin tumors.

Tumor masses were not observed protruding from the spleen, liver, or kidneys, which does not comply with the observations of (19). This may reflect that tumors are either in the growing process or are not appearing in these visceral organs due to the vaccine effect. This agrees with many pieces of literature (4,20-22) that describe the potency of vaccine administration in restricting tumor formation in MDV clinical cases. After all, only a small ratio of visceral tumors was recorded within the abdominal cavity.

These observations are variable in intensity among different clinical investigations as they reflect different pathological stages of infection. Usually, the spleen and liver

are enlarged and hyperplasia after the initial cytolitic phase, where the virus targets these lymphoid and blood-collecting organs for initial replication and invasion of T lymphocytes where the virus spends its infinitive life cycle, agree with (23).

Isolation of Marek's virus on cell culture

Virus isolation is the gold standard in the diagnostic cascade (24). It allows the investigation of the viral cytopathic effect on the target tissue, and the whole procedure yields a significant quantity of virus ready to undergo other complementary diagnostic protocols within serological and molecular techniques to characterize the target virus further or even for vaccine production (25,26).

Moreover, other studies and recommendations by (1,27,28) that described chicken kidney cells (CKC), chick embryo kidney cells (CEKC), and duck embryo fibroblast (DEF) as alternatives to CEF for the isolation of Marek's disease virus, this study utilizes CEF cells for the isolation of MDV for several reasons amongst the availability of fertile chick eggs, low growth and maintenance requirement, and high expertise in their preparation. However, one of the disadvantages of their use was the lack of Specific pathogen-free eggs, which means that these eggs may come from MDV-vaccinated parent flocks with high titer of maternal antibody that may interfere with virus isolation (29). In this study, CEF cells proved to be a suitable medium for the cultivation and isolation of MDV since the first passage with significant cytopathic effects cell rounding, cell clusters, and Syncytia matching the findings of other researchers (1,17,30,31).

However, based on PCR results, the virus titer was still low after the first passage, and that was clear with low titers of S1 and S2 compared with the positive control. This reflects the need for more passages to obtain a higher viral titer, as described by (27,32).

Confirming cell culture results

Usually, the cytopathic effect of cell culture gives an initial impression of a pathogenic virus within the inoculated sample. It reflects the need for an additional toll to confirm the viral type. In this study, molecular technique represented by real-time PCR was selected after the recommendation of (33). Taqman chemistry was designed by (15) as a highly sensitive tool to detect the pp38 gene of field virulent Marek's virus and not of the non-virulent strains. This criterion was useful when analyzing the experiment results as it showed that the tested samples S1 and S2 give a Ct value of 33 and 36, respectively, in comparison with the more concentrated positive control that read a Ct value of 24.8, as well as S3 sample that was extracted from the supernatant gives only a trace Ct value of 38.5 which ensure the sensitivity of these primers and the scientific fact that Marek's virus is a strict cell-associated virus as mentioned by (34), and agreed with (35) that MDV does not release cell-

free enveloped virus into the supernatant when grown into tissue culture cells.

Detection of virus pathogenicity by conventional PCR

The results of this study in detecting the 132bp repeats indicated that the Marek's Disease virus isolates of this study are among the highly pathogenic strains based upon the amplification of only one band with the specific primers as agreed with (36). These results were analyzed based on scientific explanations that the attenuation process through multiple virus passages in cell culture will lead to the increase in the copy number of the 132bp repeat region of Marek's Disease virus genome as referred by (37) which gives a good indicator if the virus was still highly pathogenic with limited copy number of 132bp repeat or virus attenuation with increased 132bp repeat copy number that will appear clearly after amplification and visualization on agarose comply with (30) who found that nonpathogenic strains may show 6-8 bands of the 132bp repeat.

Conclusions

This study described the isolation of Marek's Disease virus from clinically infected chicken on cell culture and the use of molecular tools, real-time PCR, and Conventional PCR to diagnose and detect 132 bp tandem repeat for the study of the virulence of the isolated strain, respectively. The results indicate that even with the continuous use of vaccination to control Marek's disease, the disease is still active and circulating in layers' farms with high pathogenic strains, requiring revised quality of vaccines, vaccinators, and management precautions to avoid outbreaks. However, vaccination is still a perfect option to restrict the oncogenesis nature of the disease. Molecular techniques provide a very accurate tool for the fast detection and pathogenic characterization of viruses, including Marek's virus.

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Conflict of Interest

The authors declare no conflicts of interest regarding this manuscript's publication and/ or funding.

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العزل والتشخيص الجزيئي لفايروس مرض الميرك من حقول الدجاج البياض في العراق

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فرع الاحياء المجهرية، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

مرض الميرك أحد اهم امراض الدواجن المسببة للأورام والتثبيط المناعي، ينتشر في انحاء العالم مصيباً الطيور بمختلف اعمارها ومسبباً خسائراً اقتصادية ملموسة يسبب المرض فايروس من نوع الحلا (الهربس). تضمنت هذه الدراسة عزل فايروس الميرك لأول مرة في العراق من ثلاثة عينات مرضية (الكبد والطحال وحوصلات الريش) والتي جمعت من حقول الدجاج البياض المشتبه اصابها بالمرض. اظهر الفحص العياني للعينات المرضية تضخم هذه الأعضاء مع وجود بقع تنخريه وأخرى نزفية على السطح، كما لوحظ وجود افات مميزة للمرض متمثلة بتضخم حوصلات الريش و تم تحديد وجود الأورام السرطانية في بعض الحالات. استخدمت الخلايا الليفية لجنين الدجاج النامي لغرض عزل الفايروس، وتم حقن العينات الفيروسيّة المحضرة على هذه الخلايا ولتميرتين. أظهر الحقن على خلايا الزرع النسيجي مجموعة من التأثيرات الرئيسية كاستدارة الخلايا وتكثفها بشكل عناقيد وتكوين الخلايا العملاقة بالإضافة الى تكوين الفجوات، ابتداءً من اليوم الخامس للحقن. تم تأكيد عزل الفايروس من خلال تقنية تفاعل البوليميريز المتسلسل اللحظي باستخدام بادئات جزيئية للجين الفيروسي PP38 إضافة الى ذلك، درست إمراضية العزلات والعينات الفيروسيّة عبر التقنيات الجزيئية عبر فحص منطقة المترادفات الجينية ١٣٢ لجينوم فايروس الميرك. اظهر الفحص ظهور حزمة مضخمة مفردة لجميع العينات المفحوصة، مما يشير الى ان جميع الفايروسات الحقلية المنتشرة هي من النوع عالية الضراوة. سوف تدعم هذه الدراسة الجهود نحو فهم افضل لمرض الميرك في حقول الدواجن العراقية، وتسهيل تشخيص المرض للحد من تأثيراته السلبية على قطاع الدواجن في البلاد. تخلص هذه الدراسة الى عزل الفايروس المسبب لمرض الميرك من العينات المجموعة من حقول الدواجن البياض عبر تمريره في خلايا الزرع النسيجي/ومن ثم دراسة إمراضية العزلات عبر التقنيات الجزيئية.