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# Using Restriction-Site Variation of PCR-Amplified 18S Ribosomal RNA Gene for Phylogenetic Analysis of *Hymenolepis* spp.

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## Abstract

*Hymenolepis* spp. infections are often asymptomatic, especially in light cases. Heavy infections can induce enteritis with nausea and vomiting, diarrhea, abdominal pain, and dizziness. Genetic therapy is the most future promising trend for treatment and prevention so, a genotype map of different parasite on microorganisms must be done. A simple and rapid polymerase chain reaction/restriction fragment length polymorphisms (PCR/RFLPs) assay, using the common restriction endonucleases *Hind*III, *Bg1*I, *Eco*RI, *Ban*II, *Sac*II and *Sst*II, is described to illustrate the genetic structure of both *Hymenolepis* species. All restriction endonucleases have been used to differentiate between both species and based on ~2200 bp long sequence of the 18S ribosomal RNA gene.

*H. nana* and *H. diminuta* were undifferentiated when their 18S rRNA genes digested with HindIII restriction endonuclease. The two *Hymenolepis* were well-differentiated when their 18S ribosomal RNA genes were digested with *Bg1*I and *Eco*RI restriction endonucleases. It's clear observed that *Ban*II, *Sac*II and *Sst*II restriction enzymes could be used as a genetic marker for *H. nana* when the enzymes uniquely fragmented the 18S rRNA gene without digesting the gene of *H. diminuta*.

**Key words: Phylogeny, *Hymenolepis*, PCR/RFLPs, 18S rRNA gene.**

## Introduction

Intestinal parasites are widely prevalent in developing countries, probably due to poor sanitation and inadequate personal hygiene. It is estimated that as much as 60% of the world's population is infected with gut parasites, which may play a role in morbidity due to intestinal infections (WHO, 1987 and Gagandeep *et al.,* 1998). In addition, the common practice of keeping wild animals as pets, particularly in small villages where usually there is no appropriate medical attention, increases the risk of unrecognized transmission of common as well as new or emerging human pathogens. Two-thirds of the world's population live in the less developed countries that lack proper sanitary facilities and a safe drinking water supply, which leads to transmission of enteric pathogens. At least 750 million episodes of diarrhea occur per year in developing countries resulting in five million deaths (Snyder and Mersen 1982).

Genus *Hymenolepis*, a cyclophyllidean tapeworm, has been described as an infrequent cause of diarrhoea in humans (Beaver *et al.*, 1984).This species was first recognized in the small intestine of a boy in Cairo in 1851 by Bilharz (Al-Hussaini *et al.,* 1979). The two species of genus infecting man, namely *Hymenolepis nana* (*H. nana*) and *H. diminuta*, cause diarrhea and abdominal pain only in hosts with heavy infection. *Hymenolepis nana* is the more common of the two parasites but both

species have a cosmopolitan distribution.

*Hymenolepis nana* is easily transmitted directly from person to person. Although *H. nana* has a short life span (a few weeks only), it is easily renewed by new generations of *H. nana* as they complete their life cycle only in human intestine. *Hymenolepis nana* may cause epidemics in institutions for children. It has also been shown that, *H. nana* infection in mice is profoundly influenced by immunosuppression. This immunosuppression is caused by T-cell deprivation or by induced steroid treatment which results in increased multiplication of abnormal cysticercoids in viscera (WHO 1981). This indirectly suggests that hymenolepsiasis could be another parasitic condition which should be eliminated before initiating immunosuppressive therapy.

*H. diminuta* (rat tapeworm) is a rodent parasite for which arthropods act as intermediate hosts. Eggs ingested by the arthropods develop into cysticercoid larvae. Rodents become infected by ingesting the arthropods. Humans, usually children, can accidentally be infected through the same mechanism. Rodents, particularly rats, are the definitive hosts and natural reservoirs of *H. diminuta*. Coprophilic arthropods (fleas, lepidoptera, and coleoptera) act as intermediate hosts. When an infected arthropod is eaten by the definitive host, the cysticercoids present in its body cavity develop into an adult worm, whose eggs are passed in the stool. It has recently been reported that beetle-to-beetle transmission of *H. diminuta* occurs in natural environments and that eggs can be dispersed in the environment via beetle feces (Pappas and Barley, 1999), thereby representing a source of additional infections and a mechanism of egg dispersal.

*H. diminuta* infection in humans is uncommon (Levi *et al.*, 1987; Hamrick *et al.*, 1990 and Varghese *et al.*, 1998); only a few hundred cases have been reported (McMillan *et al.*, 1971 ; Tantalean and Caceres 1972; Stafford *et al.*, 1980; Kan *et al.*, 1981; Pampiglione *et al.*, 1987; Tesjaroen *et al.*, 1987 Cohen 1989; Lo *et al.*, 1989; Mercado and Arias 1995 and Tena *et al.*, 1998;). *H. nana* is more commonly reported as a cause of human infection since its transmission does not require any intermediate host and it can be spread directly from person to person(Foresi 1967 and Scaglione *et al.*, 1990). In developed countries, *H. diminuta* infection is very rare and is limited to rural or degraded areas.

Advances in molecular biology techniques have enabled the direct analysis of the nuclear DNA and the mitochondrial DNA of *H. diminuta* and *H. nana* for their genotype identification (Bolla and Roberts 1971; Carter *et al.*, 1972; Henderson and Hanna 1988; Asano et al, 1996; Okamoto *et al.*, 1997 and von Nickisch-Rosenegk *et al.*, 2001). *H. diminuta* and *H. nana* were identified by using the sequence of the nuclear ribosomal internal transcribed spacer 2, ITS2, (Okamoto *et al.*, 1997).

The aim of the present study was to investigate the use of restriction profiles resulting from digestion of the 18S rRNA gene with some restriction endonucleases for separation of the majority *Hymenolepis* species from Egypt, which would be a step in their genetic map for genotherapy usage.

## Material And Methods

Individuals of the tapeworm *H. nana* and *H. diminuta* were collected from infected rat intestine and preserved in saline solution. The worms were then homogenized in 150 µl CTAB

(cetyltrimethyl-ammonium bromide) buffer using two clean slides and transported to a 1.5 ml eppendorf tube, followed by addition of 450 µl CTAB buffer supplemented with 50 µg Proteinase K and incubated at 65°C for 2 h. Proteins were removed by extraction with phenol/chloroform, and the DNA was precipitated by adding 50% (v/v) 7.5 M ammonium acetate and an equal volume of 100% ethanol. After centrifugation and washing of the pellet with 70% ethanol, it was dried under a vacuum and resuspended in 50 µl $ddH_2O$ (Rogers and Bendich, 1985 and Doyle and Doyle, 1990). One µl of the resuspended pellet was checked by 0.8% agarose gel electrophoresis for the presence of DNA, as in Figure 1.

The 18S ribosomal RNA gene of the two species were detected as in Kessing *et al*. (1989) and Stohard and Rollinson (1997) by using the primers SSU1 (5′─CGACTGGTTGATCCTGC CAGTAG─3′) and SSU2 (3′─TCCTG ATCCTCTCAGGTTCAC─5′)espective ly. The program of the polymerase chain reaction for amplification of nuclear SrRNA was: 30-35 cycles; one minute, at 94°C; two to three minutes, at 45°C; and three minutes, at 72°C.

*Hind*III, *Bg1*I, *Eco*RI, *Ban*II (Sigma-Aldrich), *Sac*II (Boehringer Mannheim) and *Sst*II (Life technologies Inc.) restriction endonucleases were used to identify and to differentiate the 18S rRNA gene of the two strains of *Hymenolepis* species. For each digestion reaction, One µl was used together with 1.2 µl of the particular enzyme buffer for a final volume of 12.2 µl. The digestion was performed for ~3.5 h at ~37°C, and the digestion products were evaluated on 2% TBE-agarose gels and stained with ethidium bromide. Restriction patterns were detected upon ultraviolet transillumination and photographed (Awwad and Morsy, in press).

## Results

The nuclear 18S rRNA genes were detected for the two *Hymenolepis* strains from the PCR products. The nuclear 18S rRNA genes sizes were approximately 2200 bp (Figure 2).

The two species of *Hymenolepis* did not differentiate when their 18S rRNA genes were digested with *Hind*III restriction endonuclease (Table 1 and Figure 3: lanes 1 and 2). The result of *Hind*III digestion of the *H. nana* and *H. diminuta* PCR products gave two restriction fragments for both (~150 and ~2050 bp; Table 1 and Figure 3: lanes 1 and 2).

*Bg1*I and *Eco*RI restriction enzymes differentiated between the two species of *Hymenolepis* (Tables 2 and 3; Figures 4 and 5). *Bg1*I restriction endonuclease cut the 18S rRNA gene of *H. nana* into two restriction bands (~950 and ~1250 bp; Figure 4: lane 1). The same restriction enzyme fragmented the 18S rRNA gene of *H. diminuta* into two fragments (~900 and ~1300 bp; Figure 4: lane 2). The two species of *Hymenolepis* were differentiated when their 18S rRNA genes were digested with *Eco*RI restriction endonuclease (Figure 5 and Table 3). *Eco*RI restriction enzyme gave three restriction fragments (~50, ~200 and ~1950 bp; Figure 5: lane 1) when digested the 18S rRNA gene of *H. nana*. Whenever, the same restriction enzyme digested *H. diminuta* 18S rRNA gene into two cuts (~300 and ~1900 bp; Figure 5: lane 2).

The 18S rRNA gene of *H.nana* species were digested uniquely with *Ban*II, *Sac*II and *Sst*II restriction endonucleases without digesting of the 18S rRNA of *H. dimintuta* (Figures 6, 7 and 8; Tables 4, 5 and 6, respectively). *Ban*II restriction endonuclease digested the PCR product of *H. nana* into two restriction fragments (~200 and ~200 bp; Figure 6: lane 1) without cut the 18S rRNA gene of *H. diminuta* (lane 2).

*Ssc*II enzyme gave two restriction bands when digested the 18S rRNA gene of *H. nana* (~600 and ~1600 bp; Figure 7: lane 1) without any fragment with the 18S rRNA gene of *H. diminuta* (lane 2). Also, *Sst*II restriction endonuclease digested *H. nana* 18S rRNA gene uniquely into two restriction fragments (~650 and ~1550 bp; Figure 8: lane 1) without digestion of the 18S rRNA gene of *H. diminuta* (lane 2).

**Table 1: Shows the length of 18Sr RNA genes fragments, resulted from digestion with *Hind*III enzyme in the two *Hymenolepis* species.**

| *Hymenolepis* strain | Band 1 | Band 2 | Band 3 |
|---|---|---|---|
| *H. nana* | ~150 | ~2050 | ------ |
| *H. diminuta* | ~150 | ~2050 | ------ |

**Table 2: Shows the length of 18S rRNA genes fragments, resulted from digestion with *Bg1*I enzyme in the two *Hymenolepis* species.**

| *Hymenolepis* strain | Band 1 | Band 2 | Band 3 |
|---|---|---|---|
| *H. nana* | ~950 | ~1250 | ------ |
| *H. diminuta* | ~900 | ~1300 | ------ |

**Table 3: Shows the length of SrRNA genes fragments, resulted from digestion with *Eco*RI enzyme in the two *Hymenolepis* species.**

| *Hymenolepis* strain | Band 1 | Band 2 | Band 3 |
|---|---|---|---|
| *H. nana* | ~50 | ~200 | ~1950 |
| *H. diminuta* | ~300 | ~1900 | ------ |

**Table 4: Shows the length of SrRNA genes fragments, resulted from digestion with *Ban*II enzyme in the two *Hymenolepis* species.**

| *Hymenolepis* strain | Band 1 | Band 2 | Band 3 |
|---|---|---|---|
| *H. nana* | ~200 | ~2000 | ------ |
| *H. diminuta* | ------ | ------ | ------ |

**Table 5: Shows the length of SrRNA genes fragments, resulted from digestion with *Sac*II enzyme in the two *Hymenolepis* species.**

| *Hymenolepis* strain | Band 1 | Band 2 | Band 3 |
|---|---|---|---|
| *H. nana* | ~600 | ~1600 | ------ |
| *H. diminuta* | ------ | ------ | ------ |

**Table 6: Shows the length of SrRNA genes fragments, resulted from digestion with *Sst*II enzyme in the two *Hymenolepis* species.**

| *Hymenolepis* strain | Band 1 | Band 2 | Band 3 |
|---|---|---|---|
| *H. nana* | ~650 | ~1550 | ------ |
| *H. diminuta* | ------ | ------ | ------ |

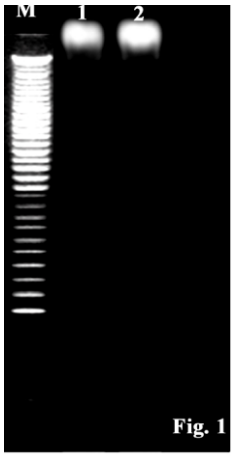

Fig. 1

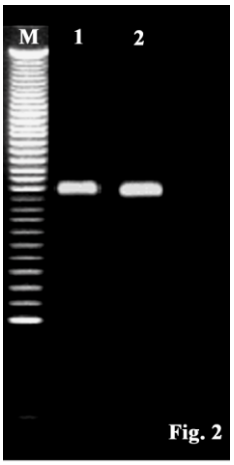

Fig. 2

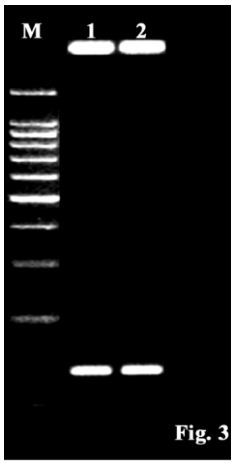

Fig. 3

Fig. 4

Fig. 5

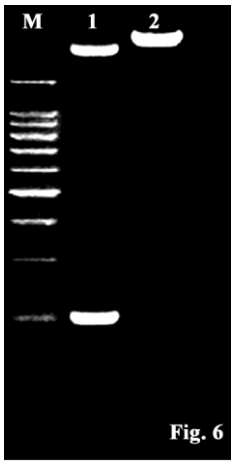

Fig. 6

Fig. 7

Fig. 8

**Figure 1:** Total DNA genome from *Hymenolepis* spp. Lane M is DNA marker (100 – 4000 bp). Lanes 1 and 2 represent the DNA of *H. nana* and *H. diminuta* respectively.

**Figure 2:** Shows full-length of 18S rRNA gene (~2200 bp) of *Hymenolepis* spp. Lane M is the DNA marker (100 – 4000 bp). Lanes 1 and 2 represent the DNA of *H. nana* and *H. diminuta* respectively.

**Figure 3:** Shows the representative 18S rRNA gene PCR/RFLPs bands from *H. nana* (lane 1) and *H. diminuta* (lane 2) with *Hind*III restriction endonuclease, which produced the same fragments (two bands: ~150 and ~2050 bp, for both). Lane M is the DNA marker ( 100 – 1500 bp).

**Figure 4:** Shows *Bg1*I restriction enzyme digested the 18S rRNA gene of *H. nana* into two restriction bands (~950 and ~1250 bp) and gave different two restriction patterns in sizes with *H. diminuta* (~900 and ~1300 bp). Lane M is the DNA marker ( 100 – 1500 bp).

**Figure 5:** Shows *Eco*RI restriction enzyme digested the 18S rRNA gene of *H.nana* into three fragments (~50, ~200 and ~1950 bp; lane 1) and the gene of *H. diminuta* into two fragments (~300 and ~1900 bp; lane 2). Lane M is the DNA marker ( 100 – 1500 bp).

**Figure 6:** Shows *Ban*II restriction enzyme digested, uniquely, the 18S rRNA gene of *H. nana* into two restriction bands (~200 and ~2000 bp; lane 1) and did not react with the gene of *H. diminuta* (lane 2). Lane M is the DNA marker ( 100 – 1500 bp).

**Figure 7:** Shows *Sac*II restriction enzyme digested, uniquely, the 18S rRNA gene of *H. nana* into two restriction bands (~600 and ~1600 bp; lane 1) and did not react with the gene of *H. diminuta* (lane 2). Lane M is the DNA marker ( 100 – 1500 bp).

**Figure 8:** Shows *Sst*II restriction enzyme digested, uniquely, the 18S rRNA gene of *H. nana* into two restriction bands (~650 and ~1550 bp; lane 1) and did not react with the gene of *H. diminuta* (lane 2). Lane M is the DNA marker ( 100 – 1500 bp).

## Discussion

The accurate identification of intestinal helminthes has important implications for many areas, including systematics (taxonomy and phylogeny), population genetics, ecology and epidemiology, and is also central to diagnosis, treatment and control of the diseases they cause. Individual cestodes are frequently identified and distinguished on the basis of morphological features, their transmission patterns or their pathological effects on the host. However, these criteria are often insufficient for specific identification (Lichtenfels *et al.*, 1997 and Andrews and Chilton, 1999). Immunological, biochemical and nucleic acid techniques provide powerful tools to overcome this limitation (McManus and Bowles, 1996 and Andrews and Chilton, 1999). In particular, the advent of the PCR method (Saiki *et al.*, 1985 and Mullis *et al.*, 1986) has revolutionized cestode taxonomy and genetics, mainly because its sensitivity permits the amplification of genes or gene fragments from minute amounts of genomic DNA.

The possibility of using molecular tools for identification of cestodes of medical importance has contributed to increased knowledge of the genus *Hymenolepis*. The present study demonstrate that PCR-RFLP of the 18S rRNA gene of *Hymenolepis* spp., using *Hind*III, *Bg1*I, *Eco*RI, *Ban*II, *Sac*II and *Sst*II, permits the differentiation of the majority of the two *Hymenolepis* species examined.

*Hind*III restriction endonuclease couldn't differentiate between the two species of *Hymenolepis* when digested their 18S rRNA genes and gave the same restriction profiles. *Hind*III restriction enzyme cut the genes of *H. nana* and *H. diminuta* into similar two restriction fragments. The results of *Hind*III restriction enzyme digestion may be proved that the two species of *Hymenolepis* have phylogenetic similarity.

*Bg1*I and *Eco*RI restriction enzymes identified and differentiated *H. nana* and *H. diminuta* when cut their 18S rRNA genes into different fragments. *Bg1*I restriction endonuclease digested the genes of *H. nana* and *H. diminuta* into two different PCR/RFLPs profiles. Also, the 18S rRNA genes of *H. nana* and *H. diminuta* were digested with *Eco*RI restriction enzymes and gave three restriction fragments with *H. nana* and two fragments with *H. diminuta*. The previous results indicated that the two species of *Hymenolepis* are intra-specific different and polyphylogenetic relationship.

Uniquely, *Ban*II, *Sac*II and *Sst*II restriction endonucleases digested the 18S rRNA gene of *H. nana* without digestion of the same gene of *H. diminuta*. Then, *Ban*II, *Sac*II and *Sst*II restriction endonucleases could be used as molecular marker for identifying *H. nana*.

The sequences of 18S rRNA genes of the *Hymenolepis* species will be analyzed in the future to better understand the intraspecific and interspecific relationships among this species (Okamoto *et al.*, 1997 and von Nickisch- Rosenegk *et al.*, 2001).

The present study shows here that PCR-RFLP is a simple and rapid technique representing an important advance for studies of *Hymenolepis* species which can be used as a step in genotherapy in the future. The study demonstrated that 18S rRNA gene contains useful genetic markers for the genotype identification of these organisms. Also, the results obtained with PCR-RFLP are concordant with the actual morphological systematics proposed for the *Hymenolepis* species by Beaver *et al.*, (1984). Future investigation must be done to investigate new method for protection of these species.

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# استخدام التغاير فى أماكن القطع للجين 18س الريبوزومى الناتج بطريقة التفاعلات المتتابعة لإنزيم البلمرة لمعرفة العلاقات التطورية لجنس الهيمينولييباس

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إن الطفيليات المعوية والتى تعيش فى الجهاز الهضمى بصفة عامة والأمعاء بصفة خاصة تنتشر وبصورة كبيرة فى البلاد النامية وخاصة فى مصر وذلك لقلة الإمكانيات المعملية والتشخيصية وكذلك الجهل الطبى للناس. إن جنس الهيمينولييباس يعتبر من الديدان الشريطية الخطيرة على صحة الحيوان والإنسان على حد سواء وينتمله عدة أنواع ومنها نوعان من أخطر ما يمكن وهما الهيمينولييباس نانا و الهيمينولييباس دايمينيوتا.
لقد تم إكثار الهيمينولييباس نانا و الهيمينولييباس دايمينيوتا صناعيا فى الفئران المنزلية وبالتالى تم عزلهما. ولخطورة هذه الكائنات تم الدراسة عليهم باستخدام تكنولوجيا أكثر تطورا كاستخدام تقنية التغاير فى طول القطع المحددة لجين 18س الريبوزومى للتفرقة بين السلالاتان للهيمينولييباس, والذى يختلف حسب الطراز الذى ينتمى إليه الكائن الحى.
وقد تم عن طريق الفصل الكهربى استخلاص جينات ال18س الريبوزمى من النواة بطريقة التفاعلات المتتابعة لإنزيم البلمرة. وقد وجد أن الجين أو المورث فى النواة للسلالتين عبارة عن حوالى 2200 من أزواج القواعد النيتروجينية. وكذلك تم استخدام إنزيمات القطع SacII, BanII, EcoRI, Bg1I,HindIII وSstII لتحديد أماكن قطع هذه الإنزيمات فى المورث لكلا النوعين وذلك لتعيين أوجه الاختلافات الوراثية بينهما.
وقد وجد أنه عند هضم الجين بإنزيم HindIII أن سلالاتى جنس الهيمينولييباس تعتبرا ذات أصل وراثى واحد. فعند هضم الجين (أو المورث) بإنزيم HindIII أعطت قطعتين (حوالى 150و 2050 من أزواج القواعد) بنفس الحجم فى النوعين(النانا والديمينيوتا).
كما اتضح أن سلالاتى الهيمينولييباس نانا و الهيمينولييباس دايمينيوتا ربما تكونا مختلفتين وراثيا أو ذات أصول متعددة حيث أنهما قد اختلفا عند هضم موروثهما بإنزيمى القطع Bg1Iو EcoRI. فعند هضم جين ال18س الريبوزمى لسلالة الهيمينولييباس نانا بإنزيم القطع Bg1I أعطى قطعتين (حوالى 950 و 1250 من أزواج القواعد) وعند هضم المورث لسلالة الهيمينولييباس دايمينيوتا بنفس الإنزيم أعطى قطعتين مختلفتين فى الحجم (حوالى 900 و 1300 من أزواج القواعد). وكذلك عند هضم المورث للسلالتين بإنزيم القطع EcoRI نتج عن ذلك ثلاث قطع (حوالى 50 و200و 1950 من أزواج القواعد) مع سلالة الهيمينولييباس نانا, وأعطى قطعتين مع سلالة الهيمينولييباس دايمينيوتا وكان حجمهما (حوالى 300 و 1900 من أزواج القواعد). وعلى هذا فربما تكونا سلالتى الهيمينولييباس نانا و الهيمينولييباس دايمينيوتا مختلفتين فى الصفات الوراثية وكذلك ذات أصول وراثية متعددة ومختلفة.
إنه من الممكن استخدام أنواع معينة من إنزيمات القطع كمجسات لتعيين أو معرفة نوع معين من نوعى جنس الهيمينولييباس. فعند استخدام إنزيمات القطع SacII, BanII وSstII لهضم جين ال18س الريبوزمى لسلالتى الهيمينولييباس نانا و الهيمينولييباس دايمينيوتا فلم يتم

التعرف إلا على سلالة الهيمينولييباس نانا. فمثلا عند هضم جين ال18س الريبوزمى لسلالة الهيمينولييباس نانابإنزيم BanII أعطى قطعتين وكان حجمهما (200 و 2000 من أزواج القواعد) ولم يهضم جين ال18س الريبوزمى لسلالة الهيمينولييباس دايمينيوتا. وقد هضم إنزيم SacII جين ال18س الريبوزمى لسلالة الهيمينولييباس نانا إلى قطعتين وكان حجمهما حوالى (600 و 1600 من أزواج القواعد) ولم يهضم جين ال18س الريبوزمى لسلالة الهيمينولييباس دايمينيوتا. وكذلك عند هضم جين ال18س الريبوزمى لسلالة الهيمينولييباس نانابإنزيم SstII أعطى قطعتين وكان حجمهما (650 و 1550 من أزواج القواعد) ولم يهضم جين ال18س الريبوزمى لسلالة الهيمينولييباس دايمينيوتا.

إن هذه الدراسة أوضحت أن استخدام تقنية طريقة التفاعلات المتتابعة لإنزيم البلمرة وإنزيمات القطع تعتبر تقنية بسيطة وسريعة لدراسة جنس الهيمينولييباس وتعتبر أداة مكملة ومتممة للتعرف الشكلى لهذه الكائنات. وقد أظهرت الدراسة أن مورث ال18س الريبوزومى يمكن استخدامه كمجسات جينية للتعرف على هذه الكائنات. و أثبتت الدراسة أن استخدام طرق البيولوجيا الجزيئية تعطى صورة تصنيفية اكثر دقة عن استخدام الطرق التقليدية.