Development of an Efficient PCR-Based Cloning Strategy for MMP9: Enabling Molecular Insights into Tumor Invasion and Metastasis

Jeevabathseba. B[1], Muruganadam. N[2], Neethirajan, G[1,2*]
[1]Division of Microbiology, ICMR-RMRC, Dolligunj, Port Blair-744103, S.Andaman
[2]Research & Development, School of Bioscience-, KG College of Arts and Science, Saravanampatti, Coimbatore-641035, TN, India

Abstract

Matrix metalloproteinase 9 (MMP9) is an important extracellular protease involved in tissue remodeling, regeneration, and disease progression. Cloning the MMP9 gene is essential to better understand its functional roles and explore its potential as a therapeutic target. The present study set out to clone the MMP9 gene from mammalian cell lines using a series of molecular biology techniques.

Methods
Immortalized mammalian cell lines were cultured under standard conditions, and total RNA was extracted. The isolated mRNA was reverse-transcribed into complementary DNA (cDNA), which served as the template for PCR amplification. Initial attempts to amplify the full-length gene in a single reaction were unsuccessful. To address this, a molecular sewing approach was implemented. The MMP9 gene was divided into smaller fragments, which were individually amplified and then fused by overlap extension PCR. The assembled full-length product was ligated into the pBOBI vector and introduced into E. coli Stbl2 cells by transformation. Confirmation of successful cloning was achieved through restriction digestion, agarose gel electrophoresis, and computational sequence analysis.

Results
Matrix metalloproteinase 9 (MMP9) plays a pivotal role in extracellular matrix degradation and cancer metastasis. This study developed an efficient PCR-based cloning strategy to enable detailed analysis of MMP9 function. Initial full-length PCR amplification attempts were unsuccessful due to complex gene structure. A molecular sewing approach was adopted, dividing MMP9 into overlapping fragments that were separately amplified and fused by overlap extension PCR. The assembled gene was ligated into the pBOBI vector and transformed into E. coli Stbl2 cells. Confirmation by restriction digestion and in-silico analysis verified cloning accuracy. This optimized workflow provides a robust foundation for future expression and functional studies of MMP9 in tumour progression and therapeutic development.

Discussion
This study demonstrates that direct PCR amplification may be insufficient when working with large or complex genes such as MMP9. The application of molecular sewing proved to be a reliable method for obtaining the full-length sequence. Cloning MMP9 establishes a foundation for further research into its role in extracellular matrix dynamics and its potential in therapeutic development. These findings highlight the value of adapting and optimizing molecular techniques to overcome technical barriers in gene cloning and functional studies.

Keywords

Matrix Metalloproteinase-9 (MMP9), PCR Optimization, Cloning, HeLa cell lines, Extracellular Matrix, Cancer Metastasis

Introduction

Cancer metastasis is a complex, multi-stage process in which malignant cells spread from a primary tumour to distant organs, contributing to disease progression and impacting diagnosis and prognosis (Step, 2006). This process involves several coordinated steps, including local tissue invasion, entry into blood or lymphatic vessels, circulation, survival in foreign environments, and eventual colonization at secondary sites (Valtista and Weinberg, 2011). A number of molecular factors regulate metastasis, including matrix metalloproteinases (MMPs), which degrade extracellular matrix components and thereby facilitate tumour cell migration and invasion (Kessenbrock et al., 2010). In parallel, epithelial-to-mesenchymal transition (EMT) enhances metastatic potential by increasing cellular plasticity and motility (Thiery et al., 2009). Understanding the regulatory mechanisms that drive these events is essential for the development of targeted therapeutic interventions.

Among the MMP family, matrix metalloproteinase 9 (MMP9) is a zinc-dependent endopeptidase that plays a crucial role in extracellular matrix (ECM) degradation. While MMP9 contributes to physiological functions such as tissue repair, immune responses, and wound healing (Nagase et al., 2006), its overexpression has been strongly associated with tumour invasion, angiogenesis, and metastasis (Kessenbrock et al., 2010). Investigating the molecular regulation of MMP9 is therefore vital in cancer research, as it may reveal novel targets for therapeutic intervention.

Polymerase chain reaction (PCR) remains an essential technique for amplifying specific genes such as MMP9. This enables further applications like cloning into expression vectors for functional studies (Mansoori et al., 2017). In this study, we focused on optimizing PCR conditions to ensure high-quality amplification of the human MMP9 gene from HeLa cells, followed by successful cloning to support downstream applications.

HeLa cells, derived from cervical cancer tissue of Henrietta Lacks in 1951, represent the first immortalized human cell line cultured in vitro (Gey et al., 1952). Their rapid proliferation and adaptability have made them a staple in biomedical research, particularly in studies related to cancer, virology, genetics, and drug development (Scherer et al., 1953). HeLa cells have contributed to several major scientific advances, including the development of the polio vaccine and innovations in cancer biology (Skloot, 2010). However, the ethical implications of their origin continue to prompt important discussions about patient consent and research ethics. In this study, HeLa cells are used as a model system for the amplification and cloning of the MMP9 gene.

PCR is widely recognized for its high sensitivity and precision in amplifying DNA sequences (Mullis and Faloona, 1987). Given the critical role of MMP9 in ECM remodelling and metastasis (Nagase et al., 2006), accurate amplification of its gene is a necessary step in assessing its function and therapeutic potential. In our work, we optimized PCR conditions—including annealing temperatures, Mg^{2+} concentrations, and cycling parameters—to ensure efficient and specific amplification of the MMP9 gene from HeLa genomic templates. This process lays the groundwork for cloning, expression studies, and functional analysis in cancer-related investigations.

Cloning of the MMP9 gene enables in-depth studies of its function in tumour progression and ECM degradation. Once amplified, the gene is inserted into an appropriate expression vector using restriction digestion and ligation (Sambrook and Russell, 2001). The resulting recombinant plasmid is transformed into competent bacterial cells for propagation and verification by colony PCR, restriction enzyme digestion, and sequencing (Green et al, 2019). After confirmation, the construct is introduced into mammalian expression systems for gene expression analysis. Techniques such as RT-PCR and Western blotting are then employed to evaluate protein levels and enzymatic activity as part of broader functional assays (Overall and Kleifeld, 2006).

This study presents a streamlined strategy for MMP9 gene amplification and cloning, contributing to the broader understanding of its regulation in cancer metastasis. By optimizing these molecular biology protocols, we aim to support future functional analyses and therapeutic explorations.

Materials and Methods

Cell Culture

Human HeLa cell lines were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine. Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 to provide optimal growth conditions.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from HeLa cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were lysed in TRIzol, and RNA was separated from the aqueous phase after chloroform extraction and precipitated using isopropanol. The RNA pellet was washed with 75% ethanol, air-dried, and dissolved in RNase-free water. RNA purity and concentration were assessed with a Nanodrop spectrophotometer (Thermo Scientific). For cDNA synthesis, 1 µg of total RNA was reverse-transcribed in a reaction

incubated at 42 °C for 60 minutes, followed by enzyme inactivation at 70 °C for 5 minutes.

PCR Primer Design and Amplification

Primers were designed to amplify the full-length coding sequence of the human MMP9 gene. The primer sequences were as follows:

Forward primer: 5'-ATGAGTCCCCTGGCGCCCCCC-3'

Reverse primer: 5'-CGATGCCCCCCCCCCCGCG-3'

PCR reactions were performed in a 25 µL volume containing 1 µL cDNA template, 0.2 µM of each primer, 200 µM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1 unit of Taq polymerase (Thermo Scientific). Amplification conditions were optimized by adjusting annealing temperatures between 55 °C and 65 °C and varying $MgCl_2$ concentrations. The final cycling parameters were: The PCR conditions included an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 55–65 °C for 30 seconds, and extension at 72 °C for 1 minute. The reaction concluded with a final extension at 72 °C for 7 minutes.

Agarose Gel Electrophoresis

Amplified PCR products were analysed by electrophoresis on a 1.5% agarose gel prepared in 1× TAE buffer and stained with ethidium bromide. Gels were run at 100 V for 45 minutes. DNA bands were visualized under UV illumination using a gel documentation system. The expected amplicon size of ~2.2 kb for MMP9 was confirmed by comparison with a 1 kb DNA ladder (Thermo Scientific).

Gel Purification and Cloning

The target MMP9 PCR product was excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen), following the manufacturer's protocol. Purified DNA was ligated overnight into the pGEM-T Easy vector (Promega) using T4 DNA ligase at 16 °C. Ligation mixtures were transformed into chemically competent Escherichia coli DH5α cells by heat shock. Transformed cells were plated on LB agar containing ampicillin (100 µg/mL), X-gal, and IPTG for blue-white screening.

Colony Screening and Sequencing

White colonies were selected and screened by colony PCR using MMP9-specific primers to verify the presence of the insert. Positive clones were further confirmed by Sanger sequencing (Eurofins Genomics) to validate the integrity of the MMP9 sequence. Sequencing data were analysed using BioEdit software to ensure that no mutations were introduced during amplification.

Results

Matrix metalloproteinase 9 (MMP9) is a critical extracellular proteinase implicated in matrix remodelling, tissue regeneration, and disease progression. The objective of this study was to clone the MMP9 gene from mammalian cell lines to support future functional characterization. Immortalized HeLa cell lines, originally derived from cervical carcinoma in 1951, were cultured and maintained under standard conditions (Figure 1).

Total RNA—including rRNA, tRNA, and mRNA—was extracted using TRIzol reagent. The mRNA was reverse-transcribed into complementary DNA (cDNA), which served as the template for PCR amplification. MMP9 gene-specific primers targeting the full 2130 bp coding sequence were designed using A Plasmid Editor (ApE) and Integrated DNA Technologies (IDT) software.

Initial PCR attempts did not produce the expected amplicon. Three reactions with varying template amounts, primer concentrations, and cycling conditions failed to yield specific products:

Reaction 1: 100 ng cDNA, 4 mM $MgCl_2$, 0.2 µM primers, standard Taq polymerase, annealing at 58 °C

Reaction 2: 250 ng cDNA, 0.4 µM primers, modified cycling with annealing at 54 °C

Reaction 3: 500 ng cDNA, higher primer concentration, Vent DNA polymerase, and gradient PCR (52–56 °C)

To overcome these amplification challenges, a molecular sewing approach was adopted. The MMP9 gene was divided into two overlapping fragments, which were amplified separately under optimized conditions:

Fragment 1: Amplified from 100 ng cDNA with 4 mM $MgCl_2$, using a gradient annealing temperature (54–58 °C). Optimal yield was obtained at 55 °C, producing a ~1 kb amplicon (Figures 2 and 3).

Fragment 2: Amplified from 250 ng cDNA under similar conditions, yielding a ~2 kb product (Figures 2 and 3).

Both purified fragments were ligated to reconstitute the complete MMP9 sequence. The full-length amplicon and pBOBI expression vector were digested with XbaI and XhoI. The resulting fragments (2 kb insert and 8.78 kb vector backbone) were separated on agarose gel and purified. Ligation with T4 DNA ligase generated recombinant constructs of approximately 10.8 kb. Restriction digestion of selected clones confirmed the presence of the 1 kb, 2 kb, and reassembled 2 kb fragments (Figure 4).

The ligation products were transformed into E. coli Stbl2 cells by heat shock. Transformants were selected on LB agar containing ampicillin (100 µg/mL). The growth of resistant colonies indicated successful cloning.

In-silico analysis performed with ApE software verified correct insert orientation and the integrity of cloning sites (XbaI: T^CTAGA, XhoI: C^TCGAG). Restriction digestion patterns obtained from plasmid DNA of positive clones were consistent with the expected construct (data not shown).

The resulting validated recombinant plasmids are suitable for transfection into mammalian expression systems, enabling downstream assays to evaluate MMP9 expression, enzymatic activity, and functional roles in extracellular matrix remodelling. Collectively, these findings establish a robust platform for investigating MMP9 in cancer metastasis and support future therapeutic research targeting its photolytic activity

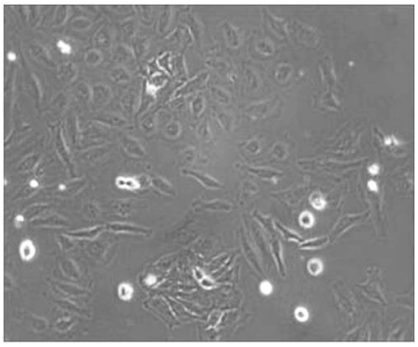

Figure-1 Microscopic image of cultured HeLa cells. The cells display characteristic epithelial morphology with adherent, polygonal shapes and prominent nuclei.

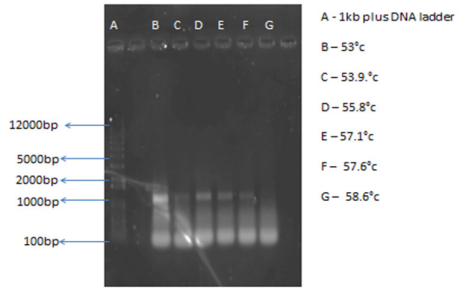

Figure 2 Agarose gel electrophoresis showing in the Gradient PCR amplification of MMP9 cDNA. A single ~1 kb band is observed, corresponding to one of the amplified MMP9 gene fragments. PCR products were resolved on a 0.7% agarose gel stained with ethidium bromide and visualized under UV light.

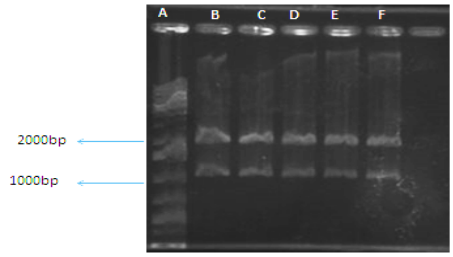

Figure 3 Amplification of 1 and 2 kb fragments in modified PCR Conditions

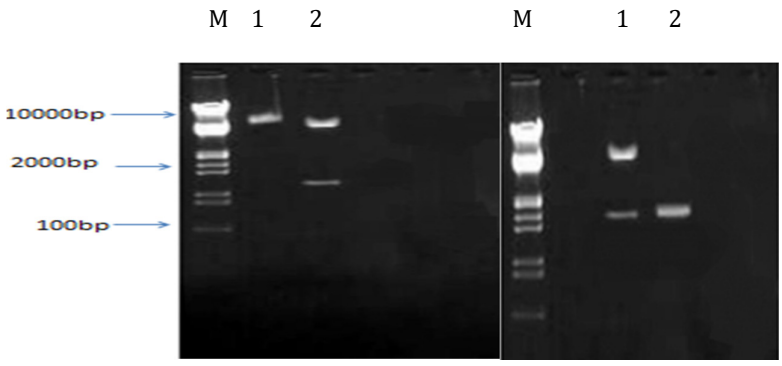

Figure 4 Restriction digestion of recombinant clone of MMP,

A- M-marker, 1-undigested cloned plasmid, 2-Digested plasmid shows the vector backbone and insert of 1kb

B- M-marker, 1--Digested plasmid shows the vector backbone and insert of 2 kb, 2- purified 2kb fragment of MMP9

Discussion

This study describes the development of an efficient PCR-based cloning strategy to generate full-length MMP9 constructs for downstream functional investigations. Matrix metalloproteinase 9 (MMP9) has been widely recognized as a pivotal factor in extracellular matrix degradation and tumour invasion (Kessenbrock et al., 2010). Its overexpression is associated with enhanced metastatic potential in multiple cancers, highlighting the importance of establishing reliable molecular tools for studying its activity and regulation.

Initial PCR amplification attempts using conventional protocols failed to produce the desired amplicons, likely due to the gene's considerable length, high GC content and secondary structures. These limitations are consistent with prior observations that large or GC-rich targets frequently yield truncated or non-specific products when amplified with standard Taq polymerase (Chuang et al., 2013). To overcome this barrier, a molecular sewing strategy was implemented, in which the gene was divided into two overlapping fragments that were separately amplified and subsequently fused by overlap extension PCR. This approach proved effective in generating a high-fidelity full-length MMP9 product, as confirmed by gel electrophoresis and in-silico sequence verification.

The use of gradient PCR to optimize annealing temperatures and magnesium ion concentrations was critical to achieving specific amplification. Optimized conditions, including annealing temperatures between 53 °C and 55 °C, facilitated the reliable amplification of both gene fragments. These results align with reports that precise control of PCR parameters is essential for reducing non-specific binding and improving yield in complex templates (Chuang et al., 2013; Saiki et al., 1988).

Following successful assembly, the full-length MMP9 gene was cloned into the pBOBI expression vector and transformed into E. coli Stbl2 cells. Positive transformants were confirmed through restriction digestion and sequencing. The availability of intact recombinant plasmids represents a valuable resource for further transfection studies in mammalian systems, where MMP9 expression and enzymatic activity can be assessed in relevant cellular contexts. This platform is anticipated to support research into MMP9-mediated extracellular matrix remodeling, tumour cell migration, and invasion (Overall and Kleifeld, 2006; Nagase et al., 2006).

Beyond its technical contributions, this work underscores the necessity of adapting molecular cloning strategies to the properties of individual target genes. While standard PCR protocols may suffice for shorter sequences, large genes often require customized approaches such as molecular sewing and rigorous optimization of reaction conditions (Green and Sambrook, 2019). The workflow established here demonstrates how these techniques can be successfully integrated to generate high-quality constructs for functional genomics applications.

Future studies will focus on expressing the cloned MMP9 gene in mammalian cell lines to evaluate its proteolytic activity, post-translational modifications, and effects on cell invasion assays. These investigations will provide deeper insight into MMP9's contributions to cancer progression and may inform the development of targeted therapeutic strategies.

Summary

This study established an efficient PCR-based cloning strategy to generate full-length MMP9 constructs, enabling future investigations into the gene's role in tumour invasion and metastasis. Standard PCR amplification failed due to the gene's length and complex structure. To address this, a molecular sewing approach was adopted, dividing MMP9 into overlapping fragments that were successfully amplified and fused by overlap extension PCR. The complete amplicon was cloned into the pBOBI expression vector, verified by restriction digestion, sequencing, and in-silico analysis, and stably propagated in E. coli Stbl2 cells. The resulting recombinant plasmids provide a robust platform for expression studies, functional assays, and therapeutic research targeting MMP9's photolytic activity in cancer progression.

References

1. Steeg, P.S., 2006. Tumor metastasis: mechanistic insights and clinical challenges. *Nature Medicine*, 12(8), pp.895–904.
2. Valastyan, S. and Weinberg, R.A., 2011. Tumor metastasis: molecular insights and evolving paradigms. *Cell*, 147(2), pp.275–292
3. Kessenbrock, K., Plaks, V. and Werb, Z., 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*, 141(1), pp.52–67.
4. Thiery, J.P., Acloque, H., Huang, R.Y. and Nieto, M.A., 2009. Epithelial-mesenchymal transitions in development and disease. *Cell*, 139(5), pp.871–890
5. Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S. and Baradaran, B., 2017. The different mechanisms of cancer drug resistance: A brief review. *Advanced Pharmaceutical Bulletin*, 7(3), pp.339–348.
6. Nagase, H., Visse, R. and Murphy, G., 2006. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research*, 69(3), pp.562–573.
7. Gey, G.O., Coffman, W.D. and Kubicek, M.T., 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Research*, 12(4), pp.264–265.
8. Scherer, W.F., Syverton, J.T. and Gey, G.O., 1953. Studies on the propagation in vitro of poliomyelitis viruses. *Journal of Experimental Medicine*, 97(5), pp.695–710.
9. Skloot, R., 2010. *The Immortal Life of Henrietta Lacks*. New York: Crown Publishing Group.
10. Mullis, K.B. and Faloona, F.A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, 155, pp.335–350.
11. Sambrook, J. and Russell, D.W., 2001. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
12. Green, M.R. and Sambrook, J., 2019. *Molecular Cloning: A Laboratory Manual*. 4th ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
13. Overall, C.M. and Kleifeld, O., 2006. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nature Reviews Cancer*, 6(3), pp.227–239.
14. ATCC, 2019. *Cell Line Authentication*. American Type Culture Collection
15. Invitrogen, 2020. *TRIzol Reagent: RNA Extraction Protocol*. Thermo Fisher Scientific.
16. Thermo Scientific, 2021. *Taq DNA Polymerase: PCR Protocols*. Thermo Fisher Scientific.
17. Promega, 2019. *pGEM-T Easy Vector System: Cloning Protocol*. Promega Corporation
18. Chuang, L.C., Huang, Y.L., Luo, Y.L. and Chen, S.H., 2013. PCR amplification of GC-rich DNA sequences using a solvent-free system. *Analytical Biochemistry*, 433(2), pp.142–148.
19. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239(4839), pp.487–491.