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Development of Affordable Methods and Protocols for Diagnosis of Clostridium difficile Infection

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ABSTRACT

Background: Clostridium difficile infection (CDI) is a major emerging pathogen in India. Its diagnosis is crucial and gradually becoming primary demand of the healthcare system. The existing diagnostics facility provides multiple pathological techniques like Enzyme linked immunosorbent assay (ELISA), Lateral flow, Nucleic acid amplification test (NAAT), etc. which are considered as the most reliable one but due to its labour-intensive, time-consuming and complicated procedure, it has a longer turnaround time and the results are not satisfactory.

Objective: To develop and Evaluate Immunological & Molecular assays using advance proteomics targeting novel biomarkers for diagnosis of CDI.

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Methodology: The present study will focus on using advance proteomics for characterization of proteins. This will be followed by peptide designing by the Kolaskar model &antipeptides synthesis. The identified peptides/lysate will be used to develop ELISA. For molecular method, Multiplex PCR will be developed which will reduce the chance of a false-negativity.

Expected Results: We propose to use the stool & culture secretome, a cocktail of several bacterial proteins, with broad specific immune response effective for diagnosis. Such toxigenic proteins may be detected using simple ELISA based tests, which is rapid yet affordable and even have scale up capacity to be converted into rapid point of care test in the form of lateral flows. Such assays, if developed, can aid clinicians in bedside screening for CDI and initiate specific treatment in suspected culture-negative cases to avoid complications associated with empirical regimes.

Conclusion: Our study will be intended to identify novel biomarkers specific to CDI develop a rapid & affordable, point of care assay which can be easily performed in any clinical laboratory, which will ultimately be helpful in the early definitive diagnosis of CDI, which if untreated will burst out as a major health problem in developing countries.

Keywords: Clostridium difficile, Diagnosis, Biomarkers, Lateral flow, Enzyme Linked Immunosorbent Assay, Polymerase Chain reaction.

INTRODUCTION

Clostridium difficile is a bacterium that can cause lethal diarrhea and infection of colon. It iswidely known to causes antibiotic associated diarrhea after long term of hospitalization and administration of broad spectrum antibiotics, and is a growing cause of concern in hospitals worldwide (1). In all developing countries including India, C.diff is under diagnosed due to lack of awareness about this pathogen, also resulting in lack of data on incidences & impact of control and prevention measures on CDI in Indian setting (2,3). On the other hand in the western world where the control and prevention of disease is acutely monitored, 15,000–20,000 deaths are reported annually from *c.diff* infection with mortality rates 6.9% within 30 days and 16.7% anually (4,5,6).

Laboratory tests are essential for its diagnosis. The available tests are the *C.diff* culture, Lateral flow kits, Toxin/Antigen detection by EIA, and toxin gene detection by Molecular Bilogy techniques (7). Among all, the gold standard is to isolate the *C. difficile* strains (8) but difficulty in cultivating this nosocomial pathogen may be a reason for the dearth of data in India. Although being considered as the most reliable one, but due to its labor-intensive,

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demand for a sophisticated Laboratory, time-consuming and complicated procedure is not considered satisfactory. Due to these reasons many clinical and pathological laboratories adopt other alternatives like, Lateral flow (LF) kits, Enzyme immuno assays (EIA) that target toxins A and/or B or glutamate dehydrogenase (GDH) and molecular assays like PCR. But the commercial LF are expensive and out of reach of the common public. Apart from this, the reproducibility of the kit is also found to very low. Another major drawback is that this LF kit is that it detects the toxins produced by *C.difficile* together and fail to differentiate both toxin A and toxin B separately, which is a crucial part of any strain level and prevalence study. The enzyme immunoassays (EIA) on the other hand, though cheaper but has a long turn over time.Molecular methods have also been reported for the *C. diff* diagnosis, but these cannot be employed in standard pathology laboratories and are often not useful for early confirmative diagnosis of c. diff (9). These tests are not economical and require elaborate Laboratory set up.

Looking into the above mentioned problems, Our study will be intended to develop a reliable, rapid, affordable, point of care gold standard diagnostic assays which can be performed in any standard pathology laboratory (10) by targeting the culture secretome which contains cocktail of several bacterial proteins, with broad specific immune response. The circulating antibodies against such secretome can be used for effective diagnosis. Such toxigenic protein may be detected using simple ELISA based test which are rapid yet affordable and even have scale up capacity to be converted into rapid point of care in form of lateral flows. Such assays if developed can aid clinicians in bed side screening of CDI. We therefore propose to develop a affordable and rapid immunodiagnostic and molecular based tests using culture filtrates, stool supernatants, designed peptides & primers for clostridium difficile.

EXPERIMENTAL DESIGN

The planned study type will be an observational study (prospective and retrospective) carried out for participant recruitment. Considering the prevalence of 10% & using a two stage sampling approach by R programming, a sample size of 311 will be sufficient to determine true estimates out of which suspected patients will be 161, Confirmed cases will be 75 and Control cases should be 75. The main theme of the study is mentioned in Fig 01.

During participant selection, inclusion criteria will be to select adults aged from 18 to 70 years Non HIV, Non hepatitis B or C negative, and non pregnant females, 3 or more loose stools in 24 hours accompanied by other gastrointestinal symptoms such as nausea, vomiting, abdominal cramps, tenesmus, bloody stools, or fever (oral temperature ≥38°C). The major exclusion criteria for this group will be any individual with a known noninfectious cause of diarrhoea such as inflammatory bowel disease.

Sample collection & processing:

Stool samples will be collected from all recruited Rural & Urban participants with diarrhoea, i.e., watery, loose stool. The sample should be fresh will be collected in a clean, dry disposable container. Parallel blood samples will be also collected wherever possible in sterile Plain vacutainer tubes.

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OBJECTIVES:

1. Identify and Characterize protein Biomarkers using stool and culture samples from Diarrheal patients using advanced proteomics technology.
2. Designing of peptides &antipeptides of identified markers.
3. To Develop & Evaluate ELISA kits (Lateral flow and 96 plate ELISA assay) using identified Novel markers for *C.diff* infection.
4. To develop ELISA/ Lateral flow assays & 96 plate ELISA assays using whole cell lysate of *C.diff* culture.
5. To develop a multiplex/Duplex *C.diff* PCR kit for the Molecular-level diagnosis of *Cdiff* infection.

MATERIALS

1. One Dimensional Electrophoresis:

1. Acrylamide solution (30 %)
2. Separating gel buffer
3. Stacking gel buffer
4. Ammonium persulfate
5. N,N,N,N-tetramethyl-ethylenediamine
6. Tris-HCI
7. Glycine
8. SDS
9. Methanol
10. Acetic acid
11. Coomassiebrilliant blue
12. **Equipments:** One dimensionalgel assembly

2. Two Dimensional Electrophoresis:

1. Urea
2. [(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate
3. Dithiothreitol
4. Tris-HCI
5. Glycerol
6. Sodium DodesylSulphate
7. Immobilized pH gradient strips pH 4–7
8. IEF Focusing tray
9. Electrode wicks
10. Blotting filter papers
11. Mineral oil
12. Forceps, pipette
13. Strip plate
14. Plastic wrap
15. SDS-PAGE protein dye
16. Methanol

17. Acetic acid
18. High purity water
19. **Equipments:** Two dimensionalgel assembly

3. Electro elution:

1. Phosphatebuffered saline
2. Whole gel eluter system

4. LC-MS/ MS Analysis:

1. Methanol
2. Tris-HCl
3. Trypsin
4. Ammonium bicarbonate buffer,pH 8.5
5. Acetic acid
6. Acetonitrile
7. Formic acid
8. HPLC grade water
9. **Equipments:** Liquid Chromatography with tandem mass spectrometry

5. Culturing:

1. Cycloserine-Cefoxitine-Fructose agar
2. Nutrient Broth
3. Brain-Heart-Infusion broth
4. Sheep blood agar plate
5. Crystal Violet Staining Reagent
6. Gram's Iodine
7. Ethanol (v/v)
8. Safranin
9. **Equipments:** DG250 Anaerobic Chamber

6. Whole Cell Lysate:

1. BHI Broth:
2. Phosphate Buffered Saline
3. Lysis Buffer (Thermofisher)
4. Acetone (Ice Cold)
5. **Equipments:** Sonicator, Centrifuge

7. Peptide & Anti-peptides Synthesis:

1. Diisopropylethylamine
2. Piperidine
3. Trifluoroacetic acid (TFA)

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4. Cold ether.
5. Acetonitrile

8. ELISA:

1. Phosphate Buffered Saline
2. Synthetic peptides
3. Bovine serum albumin (BSA)
4. Goat-anti-human IgM-horseradish peroxidase (HRP) conjugate
5. Goat-anti-human IgG-horseradish peroxidase (HRP) conjugate
6. 3,3,5,5'- tetramethylbenzidine (TMB)
7. Sulfuric acid
8. **Equipment**s: Incubator, Rotary shaker, ELISA reader, etc.

9. Molecular Biology:

DNA Extraction& PCR:

1. Lysis buffer
2. 10x Phosphate Buffer Saline (PBS)
3. Chloroform:Isoamyl Alcohol (24:1)
4. 3M Sodium Acetate
5. 70% ethanol
6. 50x TAE Buffer (Tris Acetate EDTA Buffer)
7. 10 mg /ml Ethidium Bromide solution (EtBr)
8. Buffer
9. MgCl2
10. dNTPs
11. Primers
12. DNA polymerase
13. **Equipments:** Cooling centrifuge, Vortex, Heating Block, Water bath, Thermal Cycler, etc.

METHODS

1. One- and two-dimensional polyacrylamide gel electrophoresis:

Stool & Culture samples of C.diffextracted from CDI cases will be processed for 1 D SDS PAGE. This will bedoneusing a vertical slab electrophoresis.Running gel (10%) &Stacking gel (4%) will be used as shown in Table 2.Electrophoresis will beperformed at 250 volts/50 mAmps. Gels will then be stained with Coomassie blue and the profile of novel protein will be further analysed. Band width will beevaluated using amolecular weight markerand partially purified proteins will be obtained which will then be precipitated and hydrated using ammonium bicarbonate. Each single sample will then be tranffered into the nitrocellulose paper, complexed with monoclonal antibody and observed for colour development. Samples

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with sufficient quantity of protein as mentioned in table 3,will then be proceesed by 2D PAGE. (9).

2. Electroelution:

After selection of the protein band from SDS PAGE, slice the gel portion with protein band and equilibrate in the elution buffer.Electroelute the gel in PBS and check the protein concentration. Again, run the 1D electrophoresis &resolve thepartially purified proteins and excise the band from the gel. Use the excised protein band for LC-MS/MS analysis(9).

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3. LC-MSAnalysis:

The isolated protein sample(s) will be analyzed using nano-LC with linear ion trap. The LC separation is performed with a PepMap C18 column usually at a flow rate of 400 nL/min. The LC gradient elution wil be set from a minimum to maximum limit for the mobile phase to flow and finally put back immediately after the flow is completed. An eluent will be introduced directly to the mass spectrometer via electrospray using a PicoTip emitter. Each MS scan will be followed by data-dependent MS/MS scans in triplicates for getting the accurate result(9,10).

4. Culturing:

1. Take a loopful of sample to the nutrient broth.
2. After 24 hrs of incubation in anaerobic conditions, streak a loopful of positive growth (if any) onto the CCFA & sheep blood agar plates and incubate at 37^{o}C under anaerobic conditions.
3. The positive plates will further be confirmed for its Grams nature, catalase activity followed by MALDI TOF analysis for confirmatory analysis.

Protocol for MALDI-TOF:

1. In the MS system, apply a loopful of control (E. coli ATCC 8739) in the central circle, mixed with 1 µl of Matrix solution (CHCA and Formic acid). Proper labelling and coding of the plates are to be done.
2. Take colonies from the BHI plates & apply in each circle of the slide by using 1µl of a loop or wooden toothpick followed by addition of 1µl matrix.
3. Results will be observed after the completion of the protocol.

5. Whole-cell lysate (WCL):

The pure culture of *C.diff*will be subcultured in 1 L of BHI brothand incubated at 37 °C for 4-5 days in an anaerobic Chamber. Briefly mid-log phase culture of *C.diff* (O.D ~ 0.9–1.0) will then be centrifuged&the pellet will be re-suspended in ice-cold PBSfollowed by multiple washing to ensure the removal of traces of the culture media. The pellet will then be suspended in the cell-lysis buffer and incubated at room temperature. Then few sonication cycles will be runned for breaking he cell open after which by centrifugation of the supernatant, cells will beharvested in PBS. The sample will then be subjected to protein

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quantification using Nanodrop & the resultant whole-cell antigens will belyophilised and proceesed for ELISA and lateral flow kit development(11).

6. Peptide Synthesis:

Peptidessysnthesiswill be done by Caslo Laboratories (Lyngby, Denmark) by using solid-phase methods employing N-Fmoc/t-Bu protection strategy and chemistry. Firstly the sidechain protection strategy will be employed for the standard amino acid residues. Solid-phase assembly will be carried out in the Apex396 multiple peptide synthesizer. Proper coupling cycles will be panned according to the need of the assay. Finally Fmoc deprotections will be carried out piperdine in DMF solution.

7. ELISA:

96-well ELISA technique will be developed by coatingthe flate bottom wells with around 10μg of antigen/ml diluted in PBS and incubated overnight after which the plates will begiven several washesconsecutively with PBS followed by coating with 5% BSA for 4hr. Again after a wash sample will be added to the wells and thereby coating will be completed. Sampleswill beprocessed in duplicate or Triplicates. The coated plates will be maintainedovernight at 37°C. The following day, after washing with PBS, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (1:10,000) will be added followed by tetramethylbenzidine (TMB)/ H2O2.Then 2.5 N sulphuricacidwill be added which will ingibit the reaction and the plates will be read at 450nm in an ELISA reader(12).

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8. Molecular Biology:

DNA extraction: Faecal DNA will be extracted beginning with 1 to 1.5 grams of stool samples homogenized efficiently in lysis buffer and SDS in a centrifuge tube. The content will be centrifuged in a desktop centrifuge.The supernatant will then be transferred to a centrifuge tube containing a mixture of Isopropanol and 2M Sodium acetate which will be followed by incubation at - 20^{o}C for 30 min. Centrifugation will be done and the pellet will be dried for about an hour. After this, the pellet will be dissolved in 1X Tris EDTA buffer (pH 8). Tubes will then be incubated at 65^{o}C for 15 min in moist conditions after which the entire content was transferred to a microcentrifuge tube. An approximate equal volume (0.5-0.7 ml) of Phenol: Chloroform- soamyl alcohol (24 parts choloform:1part Isoamyl alcohol) will be added, mixed thoroughly and centrifuged for 10 min at 12,000 RPM. The aqueous viscous supernatant will be carefully decanted and transferred to a new tube. An equal volume of ChloroformIsoamyl alcohol (1:1) will be added, followed bya 10 min spin at 12,000 RPM. The supernatant will be separated and then mixed with 0.6 volumes of Isopropanol to get a precipitate. The precipitated nucleic acids will then be washed with 75% ethanol, dried and re-suspended in 50 μL of TE buffer. To avoid contamination the DNA extraction protocol will be performed in a separate and isolated room and stored at -20^{o}C. The quality of DNA from the faecal samples will be tested using Nanodrop 1000 spectrophotometer (Thermo scientific). DNA concentration of the samples was determined by A260/280 measurements. Samples with a concentration more than 75ng/μL and purity in the range 1.8-2.0 were selected for further analysis (13).

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Conventional PCR:

A Multiplex PCR will be developed for the detection of multiple pathogenic toxins. The PCRs will probably as protocol mentioned in Table 04.Thermocycler conditions will be as shown in Table 05. The developed kits will be evaluated using clinical samples (stool/culture) from CDI patients and age- gender-matched controls (14-17). Few of the studies on novel diagnostic methods were repotrted (18-20). Related studies by Gupta and Bhake (21,22) and Garg et. al (23) were reviewed.

Trouble Shooting:

Sr. No.	Steps	Problem	Possible reason	Possible solution
1	ELISA	Uniform low readings	The incorrect wavelength, Insufficient washing, Insufficient development time, capture antibody did not bind to the plate.	Check filter and readers, increase development time, dilute PBS without additional proteins, re-qualify the reagents of choice.
		High Signal	Substrate solution mixed too early and turning colour uniformly.	Make fresh buffers, substrate solution to be mixed and use immediately, wash plates properly, check dilutions.
2	Lateral Flow (17)	Aggregation of colloidal gold conjugated detector antibody reagent	Improper pH of the buffer	Take care of the pH of the buffer and Antibodies which should be freshly prepared.
		Hydrophilic interactions & Hydrophobic interactions	Due to low salt conc. & using an imperfect surfactant.	Maintain the osmotic balance & use proper surfactants.
3	PCR	Carryover contamination	Contamination can be carried over from previous PCR due to aerosols, contaminating pipettes, surfaces, gloves and reagents.	This can be reduced by unidirectional workflow, Proper cleaning of pipettes, making frequent and small aliquots of reagents, aseptic cleaning techniques, proper separation of reagents stored together.
		Low purity	The purity of DNA affected	Use of purification kits, remove traces of contaminants like phenol, EDTA, Proteinase k with chemical or enzymatic DNA purification protocol, re-wash the DNA with 70% ethanol to remove salts and ions.

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Index test:

1. ELISA:

The present study proposes to evaluate the response of IgG& IgM antibodies against the pathogenic factors of clostridium difficile (Toxins & GDH). This will be done by 2 different protocols:

I. Stool samplecollected from suspected CDI cases will be subjected to 1 Dimensional electrophoresis which will be carried outusing a vertical slab gel electrophoresis system, which will be followed by Eltroelution and characterization of proteins by LCMS followed by peptide synthesis which will be done by Geneic bio and finally a kit for ELISA will be developed.

II. Targeting culture secretome using cell lysate/culture filtrate, ELISA will be developed.

2. Lateral Flow cassettes:

Using lateral flow kits operate on the same principles as the ELISA, its essence being its rapidity and affordable diagnostics, we propose to develop it targeting multiple toxin genes of Clostridium difficile (multiple toxins) in a single kit assay. This will remove the chance of missing CDI cases and improve quality diagnosis. After the basic study at CIIMS, Nagpur, the kits will be developed by Biotrol, India.

3. **Molecular Diagnosis (PCR):**

Molecular diagnostics is an outcome of successful interplay among laboratory medicine, genomics knowledge, and technology in the field of molecular genetics, especially with significant discoveries in the field of molecular genomic technologies. The above factors then contribute to the identification and characterization of the genetic basis of inherited diseases which, in turn, is vital for the accurate provision of diagnosis. We here propose to target the maximum of the pathogenic toxins in one test assay by developing a multiplexing PCR kit. This will enable us to detect and analyze several toxigenic genes in parallel, providing us with the following advantages: 1. saves cost for reagents, consumables and labour. 2. Saves time by allowing you to read dozens, even hundreds of data points simultaneously. 3. It also saves our precious sample.

Validation:

To ensure the accuracy of the reported results, each minute step of the testing process must be carefully evaluated and monitored for each test being performed and to compare the results to another standard testing technique. Such validations are relatively standardized in fields such as clinical biochemistry. We plan to get all the proposed kits validated from at least 2 different institutes who have been working on the concerned assays since long now.

EXPECTED RESULTS:

1. **Participants Information:** The participants will be recruited based on the Inclusion criterion mentioned above. Each of the participants should fill in their medical history and

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give their consent for the study by signing the consent forms. All these baseline characteristics as mentioned in Table 01, will then be tabulated for further analysis.

2. **Development of Biomarkers:** In the present study, culture secretome will be used, combined with advanced proteomics and novel protein biomarkers will be identified concerningclostridium difficile infection. Host immune response markers, if expressed, will also be studied & identified. Also, a comparative biomarker analysis will be initiated using the samples from participants testing negative for clostridium infection.

3. **Tests to be developed**: Despite the average incidence of antibiotic-associated diarrhoea in India (Limited study available), we lack definitive diagnostic tools that can aid in early diagnosis of this infection. Currently, available rapid test such lateral flow assay, ELISA kit and qPCR kits, suffer for sensitivity and specificity limitation, costly affair and non-user friendly techniques, often also fails to discriminate from true positive from false-positive cases. We propose that culture secretome of the organism can be used as an effective tool to aid in early diagnosis of diarrhoea due to heavy antibiotics. Such culture secretomes contain a cocktail of several bacterial proteins, with broad specific immune response and can be used for effective diagnosis. Such toxigenic protein may be detected using simple ELISA based test which is rapid yet affordable (Fig02) and even has scaled up capacity to be converted into rapid point of care in form of lateral flows (Fig 03). Such assays if developed can aid clinicians in bedside screening for *C.difficile* infection and initiate specific treatment in suspected culture-negative cases to avoid complications associated with empirical regimes. Currently, automated culture-based assays are available which are specific but time limiting as they depend on the final characterization of the organism by highly complicated detection instruments. We, therefore, propose to develop an affordable and rapid immunodiagnostic and molecular-based tests using culture filtrates, stool supernatants, designed peptides & primers for clostridium difficile (Fig 04). These molecular-based tools since based on proteins and DNA can be used to provide rapid detection in *C.difficile* infectious cases.

CONFLICT OF INTEREST:

None

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Figures and Tables:

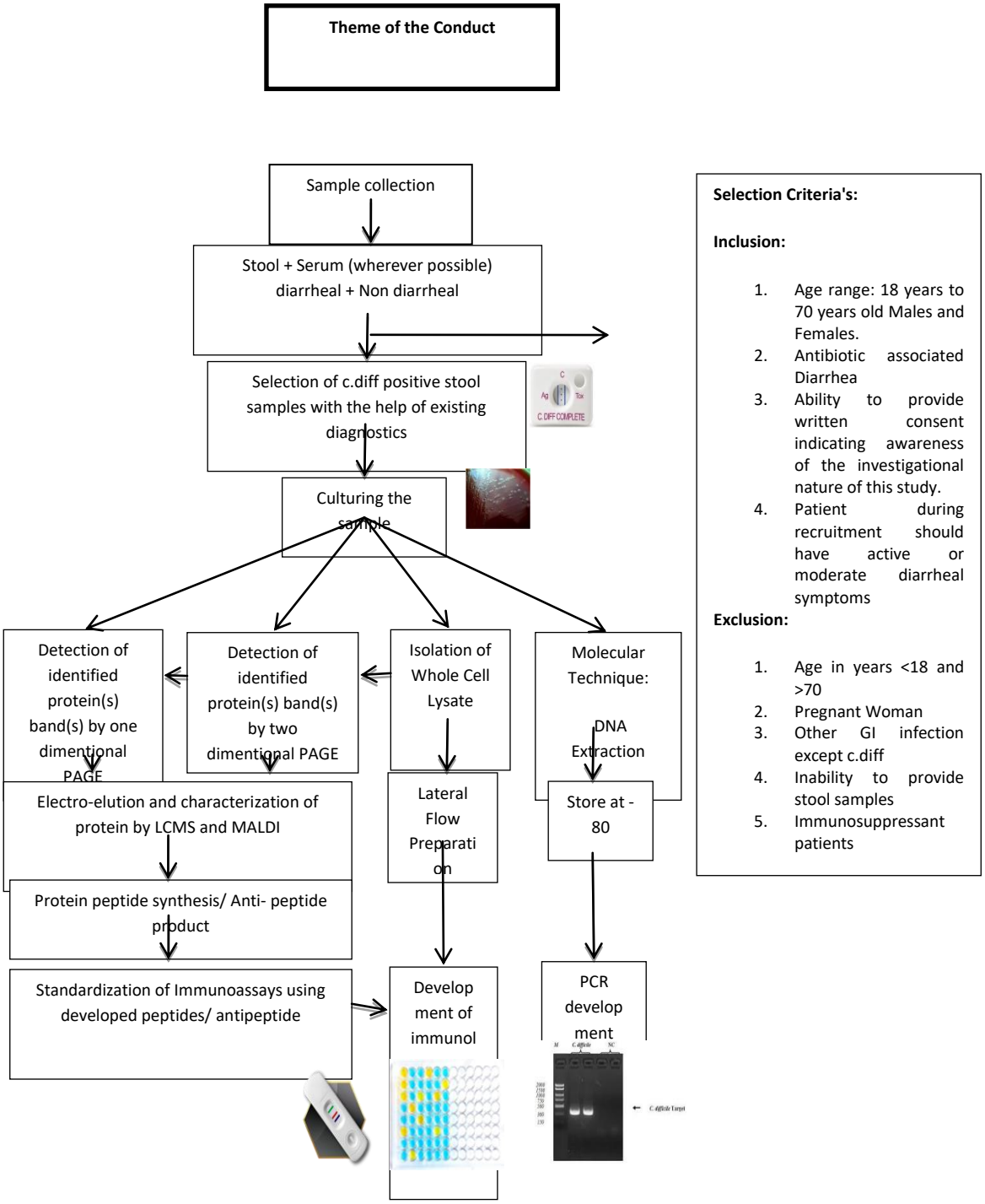

Fig 01: Schematic Representation of the Work Flow opted for the study

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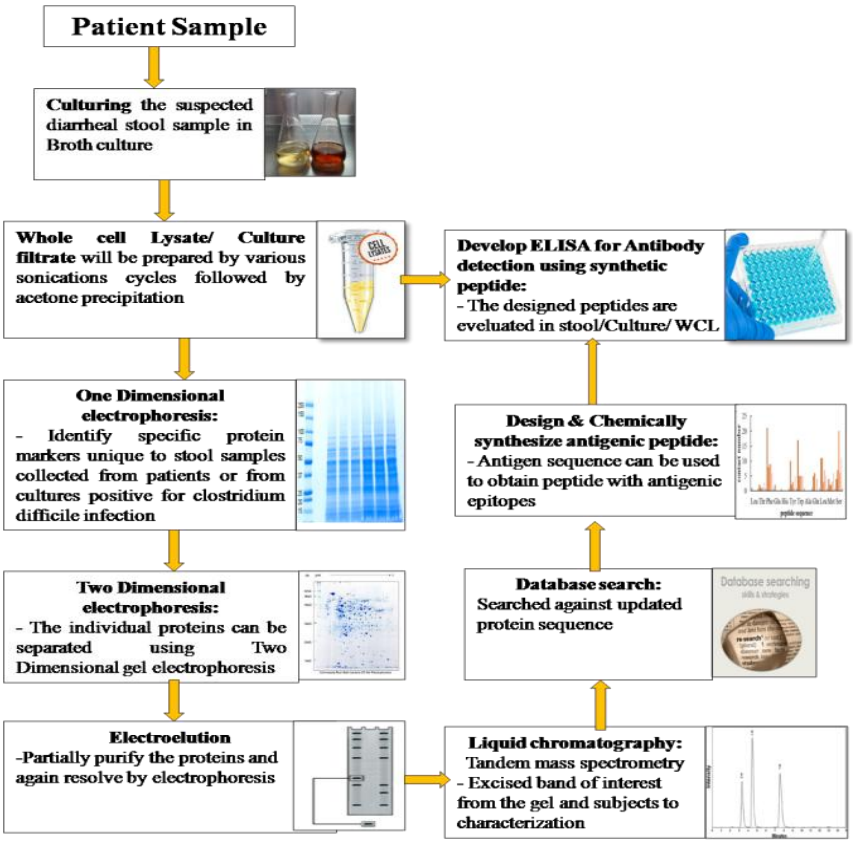

Fig 02: Schematic Overview of the expected outcome for Identification of specific protein and development of an ELISA assay through 2 different methods.

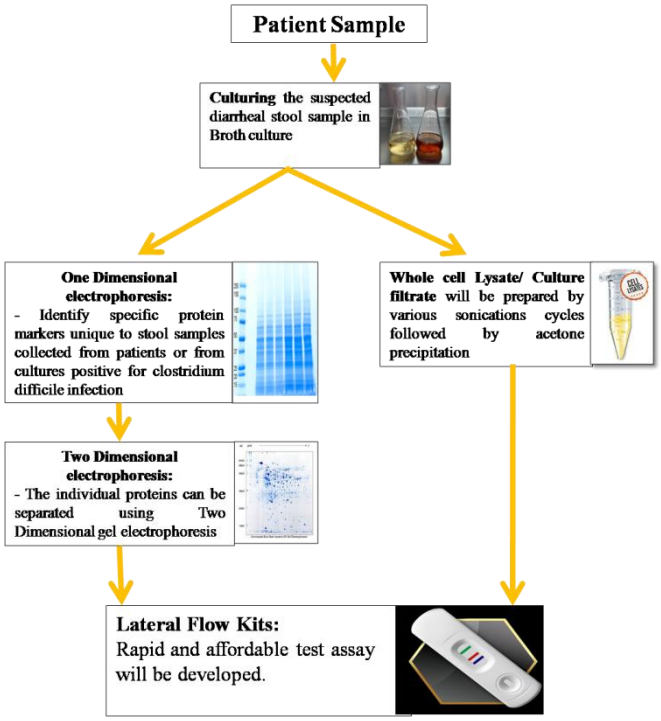

Fig 03: Schematic Overview of the expected outcome for developing Lateral Flow kits.

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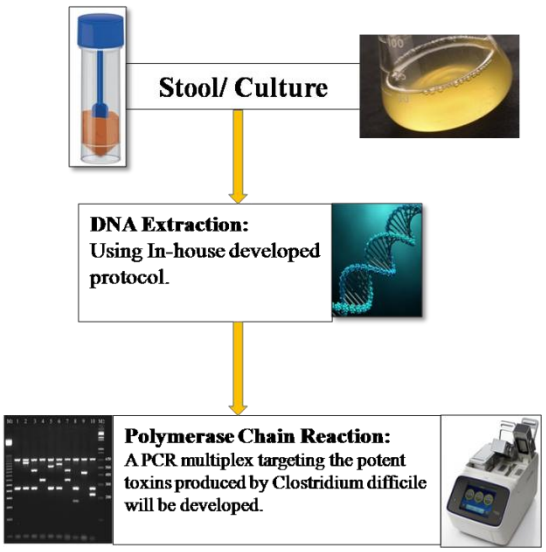

Fig 04: Schematic Overview of the expected outcome for developing a Molecular Technique.

Table 01: Characteristics of Patients Included in the Study

Characteristics	**All patients**	**Rural**	**Urban**
Age (mean)			
Gender (Male / Female)			
Pets/Animal Exposure			
Immunosuppression			
Antibiotics			
Co-morbidities			
Fever			
Vomiting			
Abdominal Pain			
Duration of Diarrhea			
No			
1-3 days			
>3 Days			
Previous History of any Bowel Infection			
Hospitalisation duration			
No			
1-3 days			
>3 Days			
BMI (mean)			
Housing condition			

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Very Good			
Good			
Average/ Moderate			
Poor			
Toilets			
Good and Clean			
Average			
Common Toilets			
No toilets			
Seasons			
Monsoon			
Winter			
Summer			

Table 02: Protocol for preparation of SDS PAGE:

	Running Gel	**Stacking Gel**
Acrylamide monomer	1.7 ml	0.5 ml
Separating Gel Buffer	1.3 ml	-
Stacking Gel Buffer	-	0.4 ml
Distilled Water	1.9 ml	2.1 ml
Mix and degas for 5min before adding the following		
SDS (10%)	50µL	30µL
APS (10%)	50µL	30µL
TEMED	10µL	10µL

Table 03: Dimensions of 2 dimensional Electrophoresis:

Strip length	**7cm**	**11cm**	**17cm**
Sample Volume (max)	125µL	185µL	300µL
Protein loaded (max)	169µg	250µL	405µL

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Table 04: Protocol for preparation of PCR Mastermix

Reagents	Concentration
PCR Buffer	1X
Tris-HCl	50 mM
KCl	10 mM
(NH4)2SO4, pH 8.3	5 mM
MgCl2	2.6 mM
dATP, dCTP, dGTP and dTTP	260µM
Taq polymerase	1.25 U

Table 05: Protocol for PCR reaction

Sr No.	Steps	Temperature	Time	cycles
1	Initial Denaturation	94°C	10 min	1
2	Denaturation	94°C	1 min	35
	Annealing	55°C	1 min	
	Extension	72°C	2 min	
3	Final Extension	72°C	10 min	1
4	Hold	4°C	∞	

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