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RESEARCH ARTICLE

USE OF RECOMBINANT TRYPSIN IN PRODUCTION OF VACCINE AGAINST MEASLES AND RUBELLA

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ABSTRACT

Trypsin is a proteolytic enzyme routinely used as an effective cell dissociating agent in tissue culture based viral vaccine manufacturing processes. Conventionally a porcine or bovine origin trypsin is used for this purpose. Being an animal origin material, it can potentially contaminate the final product by known or unknown adventitious agents inherent in the source material. Animal-derived materials are now subject to more stringent regulations. Therefore, it is essential to design production processes in such a way, that they offer maximum viral clearance potential from animal origin products. Use of animal-component-free recombinant trypsin appears to be a viable alternative for the animal origin trypsin. Two types of commercially available recombinant trypsins were selected for evaluation purpose in comparison with conventional porcine-origin trypsin. All the three trypsin preparations were used for serial passaging of human diploid (MRC-5) cells, and subsequent manufacturing of Measles and Rubella vaccine using above substrates. In conclusion, both the recombinant trypsin samples were found to be equivalent to conventional porcine trypsin with respect to important parameters viz. cell culture suitability and yield of Measles and Rubella viruses.

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1. INTRODUCTION

Trypsin is a proteolytic enzyme (serine protease), used as a cell dissociating agent in tissue culture techniques. As such, it becomes a critical raw material for all tissue culture based vaccines such as, Measles, Rubella, etc. Porcine trypsin is a widely used reagent in the manufacturing of biological medicinal products. It is extracted from pig pancreas and therefore, being an animal origin material, trypsin has an inherent risk of being contaminated with transmissible adventitious agents (de Oliveira *et al*., 2013; Petricciani *et al*., 2014; Harasawa and Tomiyama, 1994). *Mycoplasma hyorhinis*, one of the most common strains of mycoplasma found in cell cultures is also commonly associated with pigs (Polak-Vogelzang *et al*., 1990) and it can survive in trypsin solutions. Such risks of various adventitious agents are typically mitigated by testing the porcine trypsin for absence of the extraneous agents, however the testing methods have their own limitations and sometimes the contaminating adventitious agents may escape detection.

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As an example, recently a rotavirus vaccine was found contaminated with porcine circovirus DNA sequences originated from porcine trypsin that was used during development of vaccine (Gilliland *et al*., 2012; Li *et al*., 2012; McClenahan *et al*., 2011CFR, 2013). In order to minimize such risks of adventitious agents, recent international regulatory guidelines are becoming more and more stringent regarding the use of animal origin raw materials in the manufacturing of human biologicals. Requirements of 9 CFR (Code of Federal Regulations, USA) testing for biological origin raw materials, recommend testing of various adventitious viruses potentially originating from these raw materials. A CBER (Center for Biologics Evaluation and Research) 'Guidance For Industry', (2010) recommends additional testing of trypsin depending upon bovine or porcine origin [EMA/CHMP/BWP/814397/2011, 20 February 2014]. There are several limitations for viral detection during the production of porcine trypsin. As many as 55 porcine virus species of human host range, from 17 different families having been stated as potential contaminating viruses (Li *et al*., 2007). EMA 2013 (European Medicines Agency), guidelines recommend use of two different cell lines to test adventitious agents that could be found in porcine trypsin. These guidelines also

suggest use of animal-component free reagents, such as recombinant trypsin, in place of animal derived trypsin (Marcus-Sekura *et al*., 2011). Recombinant porcine trypsin is a genetically engineered protein expressed in suitable microorganism (*E. coli, Pichia pastoris*) and purified by high pressure liquid chromatography (Vasquez *et al*., 1989; Makrides, 1996; Yee and Blanch, 1993; Walker and Keil, 1973; Jónsdóttir *et al*., 2004; Luong *et al*., 1988). Use of recombinant trypsin as an alternative to conventional porcine origin trypsin can completely eliminate the risk of contamination by animal origin reagents. Two commercially available recombinant trypsin preparations were selected for evaluation purpose in comparison with conventional porcine origin trypsin. The *Biogenomics* make trypsin is manufactured by cloning synthetic gene of porcine trypsin expressed in *E coli* and the *Richcore* make trypsin is manufactured using recombinant yeast *Pichia pastoris* as an expression system. The criteria included in the evaluation study of these trypsins were, suitability for cell culture, suitability for manufacturing of Measles and Rubella vaccines.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Viruses

- Measles Virus: Edmonston-Zagreb strain
- Rubella Virus: RA27/3 strain

2.1.2 Cell lines

- Human diploid (MRC-5) cells for growing Measles & Rubella viruses
- RK-13 cells for titration of Rubella virus
- VERO cells for titration of Measles virus

2.1.3 Media and Foetal Bovine Serum

- Minimum Essential Medium (MEM) with Hank's salts and with L-Glutamine, (Make: Life technologies) as a base for preparation of cell growth medium and virus maintenance medium
- Foetal Bovine Serum (FBS) of Australian origin (Make: Moregate Biotech) as a supplement in cell growth medium at 10 to 12.5% concentration

2.1.4 Trypsin

- Conventional porcine origin trypsin (Make: Pangaea, Canada) as a control
- Recombinant trypsins (Make: *Biogenomics* & *Richcore*) for evaluation purpose.

2.1.5 Stabilizer

- Stabilizer containing Gelatin 2.5%, Sorbitol 5%, L-Histidine: 0.21%, L-Alanine: 0.1%, L-Arginine hydrochloride 1.6%, Tricine: 0.3% & Lactalbumin Hydrolyzate: 0.35% for viruses

2.1.6 Lab-ware & equipments

Tissue Culture Flasks (175 cm^2, BD Falcon), Roller Bottles (850 cm^2, Corning Inc.), Cell Factories (6320 cm^2, CF_{10}, Nunc), Microtitration plates (96 wells), Roller Apparatuses (Bellco Inc.), Inverted Microscope (AxioVert / PrimoVert, Carl Zeiss) and walk-in incubators maintained at the required temperature during cell growth, virus growth and virus content assay procedures.

Methods

2.2.1 Evaluation of suitability of recombinant trypsin for cell culture

Sequential passaging of MRC-5 cells' using 175 cm^2 tissue culture flaks was done using recombinant trypsin solutions from two different manufacturers as described earlier (Set-I & Set-II). Conventional Porcine trypsin solution was used as a control (Set-III). Based on the initial optimization studies, a concentration of 800 Units/ml of all the three types of trypsins was used for all further experimental work. Minimum essential medium supplemented with 10% foetal bovine serum was used as a cell growth medium. All the cultures were incubated at 36^oC for 3 to 4 days between two successive passages. Suitability was evaluated by comparing the cell counts, cell morphology and monolayer confluency obtained for cultures from all the sets at every passage. The experiment was performed in three lots to confirm consistency of results.

2.2.2 Senescence Study

Senescence is a gradual deterioration of functional characteristics of cells with their age. Human diploid (MRC-5) cells have been studied to have a life time of 50-60 doubling levels. In this study, passaging of MRC-5 cells was carried out using all the trypsin solutions till population doubling level (PDL) 60, to study the effect on the senescence of MRC-5 cells, if any. Cell morphology and monolayer confluency in the tissue culture flasks (TCF) was observed microscopically and cell counts were taken at every population doubling level.

2.2.3 Evaluation of suitability of recombinant trypsin for Measles Virus production

Cell factories with MRC-5 cells were passaged for three consecutive passages using each of the three types of trypsin preparations so as to achieve a population doubling level of cells similar to that used for vaccine manufacturing, These cell factories were further trypsinized using respective trypsin solutions (i.e. two recombinant trypsins and one conventional porcine trypsin) and the individual cell pools were infected with Measles working seed virus.

These cell pools were equally distributed in roller bottles and incubated at 36^oC temperature. Minimum essential medium supplemented with 10% foetal bovine serum was used as a cell growth medium. After two days of incubation, the cell monolayers were washed with virus maintenance medium (i.e. MEM without FBS) and incubation was continued at 32^oC with virus maintenance medium. Multiple harvests were

collected from individual roller bottle groups at pre-defined intervals. Stabilizer was added to the pooled harvests in quantities as described earlier. Representative samples from all the groups were withdrawn after every harvest for determination of virus content. The experiment was performed in three lots to confirm the consistency of results.

2.2.4 Evaluation of suitability of recombinant trypsin for Rubella Virus production

Three groups of three tissue culture flasks were made from a set of nine tissue culture flasks ITCF) prepared by seeding ten million cells per TCF from a homogenous pool of MRC-5 cells. Sequential passaging of tissue culture flasks from each group was carried out using two recombinant trypsin solutions and also a conventional porcine-origin trypsin for three consecutive passages to obtain cell factories (CF) with MRC-5 cells at a population doubling level that was used for vaccine manufacturing. Minimum essential medium supplemented with 10% foetal bovine serum was used as a cell growth medium.

After confluent growth, cell monolayers in all the nine cell factories were infected with Rubella working seed virus and further incubated at 31^{o}C with cell growth medium. After two days of incubation, cell monolayers were washed virus maintenance medium and further incubated at 31^{o}C temperature with virus maintenance medium. Multiple harvests were collected group wise from all the cell factories at pre-defined intervals. Stabilizer was added to the pooled harvests in quantities as described earlier. Representative samples from all the groups were withdrawn after every harvest for determination of virus content. The experiment was performed in three lots to confirm the consistency of results.

2.2.5 Sampling

All the virus titration samples were collected into 5 ml amber colour vials and stored frozen below –60^{o}C till use.

2.2.6 Titration methods

Cell culture infectivity dose ($CCID_{50}$) method in tissue cultures using 96 well plates was used for titration of all virus samples. Appropriate cell culture controls and reference virus preparation were run in every assay. All samples were tested in duplicate and the final virus titres values reported were an average of four. Necessary ten fold dilutions of virus were used for titration so as to get end point of virus titres. All the wells showing virus-specific cytopathic effect were marked as positive.

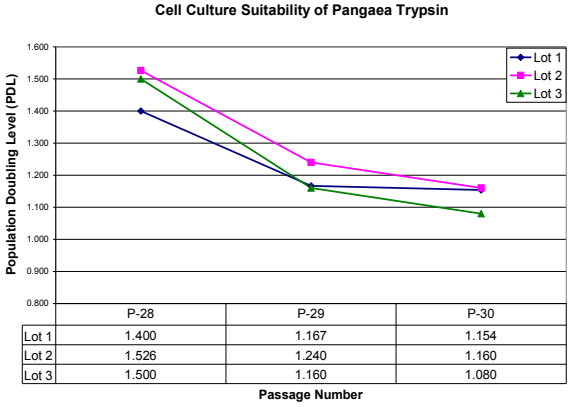
3. RESULTS

3.1 Cell Culture Suitability

Results of evaluation of suitability of recombinant trypsins for culturing MRC-5 cells are shown in Figure-3.1. Cell morphology, attachment and confluency of monolayer were studied over three successive passages done using all the three trypsins and the results were comparable.

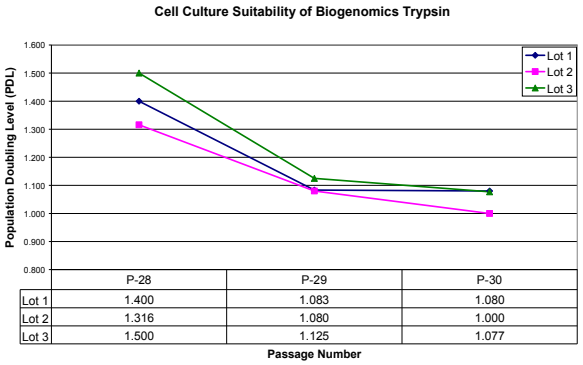
Figure 3.1.

3.1a



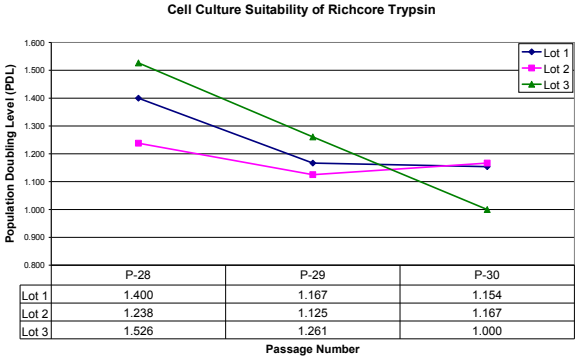
	P-28	P-29	P-30
Lot 1	1.400	1.167	1.154
Lot 2	1.526	1.240	1.160
Lot 3	1.500	1.160	1.080

3.1b



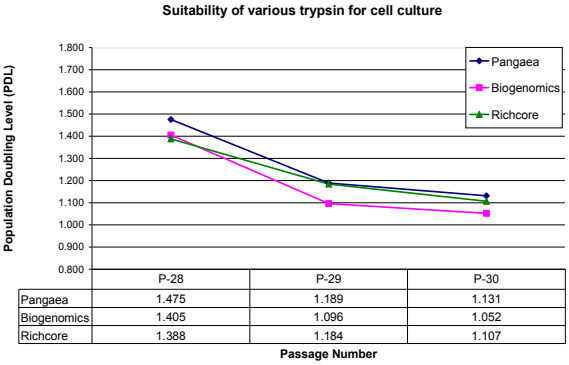
	P-28	P-29	P-30
Lot 1	1.400	1.083	1.080
Lot 2	1.316	1.080	1.000
Lot 3	1.500	1.125	1.077

3.1c



	P-28	P-29	P-30
Lot 1	1.400	1.167	1.154
Lot 2	1.238	1.125	1.167
Lot 3	1.526	1.261	1.000

3.1d



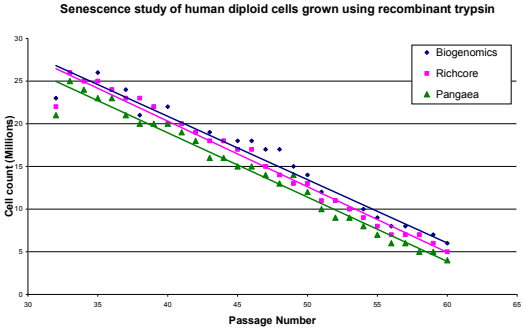
	P-28	P-29	P-30
Pangaea	1.475	1.189	1.131
Biogenomics	1.405	1.096	1.052
Richcore	1.388	1.184	1.107

Note: Cell counts are taken from 175 cm^2 TCF trypsinized after 4 days of incubation at 36±1^{o}C. In all the cases, cell morphology was fibroblastic and monolayer confluency was 100% in 4 days incubation. Last graph shows average PDL values of each type of trypsin.

3.2 Senescence Study

Cell culture suitability study was continued further till passage-60 using respective trypsin types to evaluate the effect on senescence pattern of MRC-5 cells. Figure-2 indicates the results obtained in the senescence study. As the cells grow older, the cell count of a confluent monolayer goes on decreasing with the passage number of cells. However, the pattern of cell counts up to passage-60 observed for recombinant trypsin was comparable to that of conventional porcine trypsin.

Figure-3.2



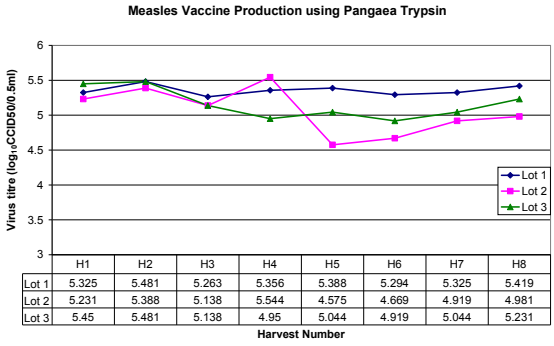
Note: Cell counts are taken from 175 cm^2 TCF trypsinized after 4 days of incubation at 36±1°C. All cell counts are expressed in millions.

3.3 Measles Vaccine Production

Figure-3.3 provides the harvest wise virus titers obtained for three Measles Production lots using each type of trypsin and a comparison of virus titres obtained from all the trypsins. It was observed that the virus titre pattern of each individual type of trypsin was consistent and was also comparable for all the types of trypsins.

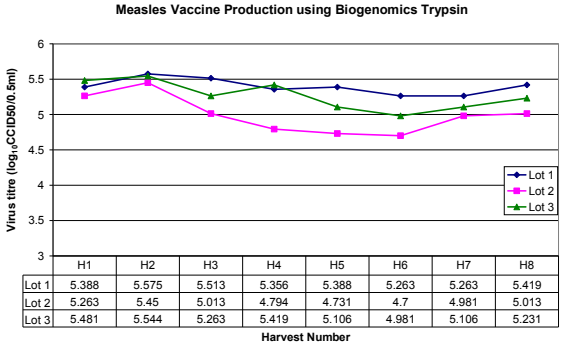
Figure-3.3

3.3a



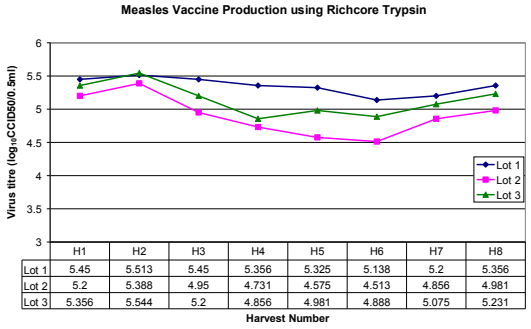
	H1	H2	H3	H4	H5	H6	H7	H8
Lot 1	5.325	5.481	5.263	5.356	5.388	5.294	5.325	5.419
Lot 2	5.231	5.388	5.138	5.544	4.575	4.669	4.919	4.981
Lot 3	5.45	5.481	5.138	4.95	5.044	4.919	5.044	5.231

3.3b



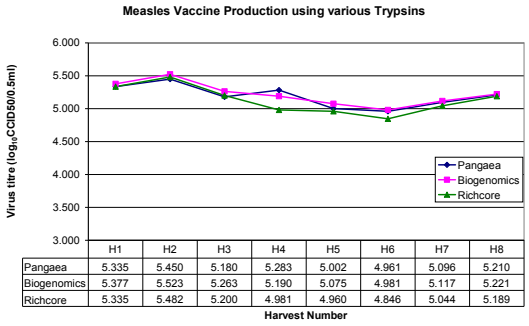
	H1	H2	H3	H4	H5	H6	H7	H8
Lot 1	5.388	5.575	5.513	5.356	5.388	5.263	5.263	5.419
Lot 2	5.263	5.45	5.013	4.794	4.731	4.7	4.981	5.013
Lot 3	5.481	5.544	5.263	5.419	5.106	4.981	5.106	5.231

3.3c



	H1	H2	H3	H4	H5	H6	H7	H8
Lot 1	5.45	5.513	5.45	5.356	5.325	5.138	5.2	5.356
Lot 2	5.2	5.388	4.95	4.731	4.575	4.513	4.856	4.981
Lot 3	5.356	5.544	5.2	4.856	4.981	4.888	5.075	5.231

3.3d



	H1	H2	H3	H4	H5	H6	H7	H8
Pangaea	5.335	5.450	5.180	5.283	5.002	4.961	5.096	5.210
Biogenomics	5.377	5.523	5.263	5.190	5.075	4.981	5.117	5.221
Richcore	5.335	5.482	5.200	4.981	4.960	4.846	5.044	5.189

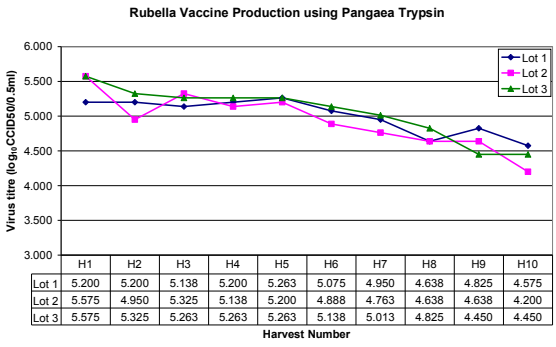
Note: All the virus titre values are expressed as $log_{10}CCID_{50}$/0.5ml. Last graph shows average virus titre values of each type of trypsin.

3.4 Rubella Vaccine Production

Figure-3.4 provides the harvest wise virus titers obtained for three Rubella Production lots using each type of trypsin and a comparison of virus titres obtained from all the trypsins. It was observed that the virus titre pattern of each individual type of trypsin was consistent and was also comparable for all the types of trypsins.

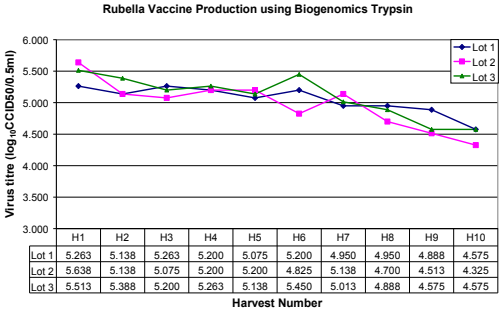
Figure-3.4

3.4a



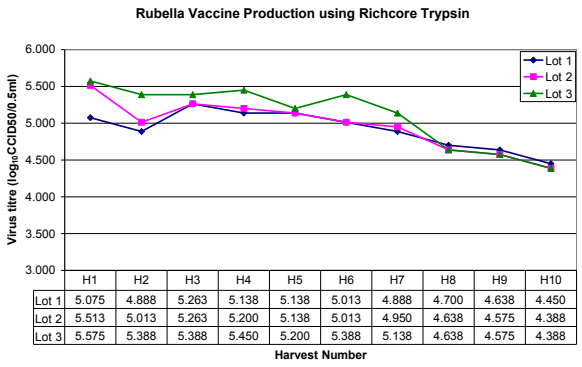
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Lot 1	5.200	5.200	5.138	5.200	5.263	5.075	4.950	4.638	4.825	4.575
Lot 2	5.575	4.950	5.325	5.138	5.200	4.888	4.763	4.638	4.638	4.200
Lot 3	5.575	5.325	5.263	5.263	5.263	5.138	5.013	4.825	4.450	4.450

3.4b



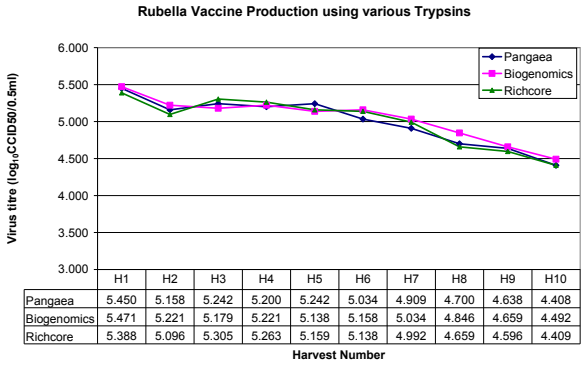
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Lot 1	5.263	5.138	5.263	5.200	5.075	5.200	4.950	4.950	4.888	4.575
Lot 2	5.638	5.138	5.075	5.200	5.200	4.825	5.138	4.700	4.513	4.325
Lot 3	5.513	5.388	5.200	5.263	5.138	5.450	5.013	4.888	4.575	4.575

3.4c



	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Lot 1	5.075	4.888	5.263	5.138	5.138	5.013	4.888	4.700	4.638	4.450
Lot 2	5.513	5.013	5.263	5.200	5.138	5.013	4.950	4.638	4.575	4.388
Lot 3	5.575	5.388	5.388	5.450	5.200	5.388	5.138	4.638	4.575	4.388

3.4d



	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Pangaea	5.450	5.158	5.242	5.200	5.242	5.034	4.909	4.700	4.638	4.408
Biogenomics	5.471	5.221	5.179	5.221	5.138	5.158	5.034	4.846	4.659	4.492
Richcore	5.388	5.096	5.305	5.263	5.159	5.138	4.992	4.659	4.596	4.409

Note: All the virus titre values are expressed as $\log_{10}CCID_{50}$/0.5ml. Last graph shows average virus titre values of each type of trypsin.

DISCUSSION

Porcine trypsin is used as a cell dissociation reagent in tissue culture technology. These preparations are nothing but acetone extracts of pig pancreas and normally contain mixture of other enzymes such as chymotrypsin, elastase, caboxypeptidase, kallikriens, and insulinase, effects of which on cell culture are not completely understood. The preparations of these acetone extracts are further mixed with lactose to adjust the potencies. This trypsin has been successfully used for last many decades for commercial scale manufacturing of tissue culture based vaccines and other biological products. However, being an animal origin material, it has a potential to introduce known or unknown adventitious agents inherent in the source material to the final product. Recently, there has been an example of rotavirus vaccine contaminated with porcine circovirus DNA sequences originated from porcine trypsin that was used during development of vaccine.

Recombinant trypsin is a genetically engineered protein expressed in suitable microorganisms (*E. coli, Pichia pastoris*) and purified by high pressure liquid chromatography (Vasquez *et al*., 1989; Makrides, 1996; Yee and Blanch, 1993; Walker and Keil, 1973; Jónsdóttir *et al*., 2004; Luong *et al*., 1988). Use of recombinant trypsin as an alternative to porcine origin trypsin can completely eliminate the risk of contamination by animal origin reagents. Recombinant trypsin preparations have been shown suitable for trypsinization of few cell lines such as Vero, CHO, etc. However, the available information is very limited.

The study in this research was aimed at evaluating possibility of use of recombinant trypsin for few cell based vaccine manufacturing processes at a commercial level. Recombinant trypsin samples from two different manufacturers were evaluated for suitability of cell culture of human diploid (MRC-5) cells with porcine origin as a control. Human diploid (MRC-5) cells are having a definite life-span, therefore, the long term effect of recombinant trypsins, if any, on senescence was also studied up to passage-60. The recombinant trypsin samples were also evaluated for suitability on virus culture using Measles and Rubella viruses.

The Biogenomics make trypsin powder has been manufactured using recombinant bacterium, *Escherichia coli* expressing the porcine trypsin gene, whereas the Richcore make trypsin powder was manufactured using recombinant yeast *Pichia pastoris* expressing synthetic trypsin gene [3,4,5,6]. The pattern of trypsinization of MRC-5 cells using both the recombinant trypsin solutions was comparable to conventional porcine trypsin. The attachment, morphology and confluency were found to be satisfactory, as observed under the microscope from time to time during sequential passaging experiments. Senescence study on MRC-5 cells, sub-cultured till passage-60, using recombinant trypsin was comparable with that obtained for conventional trypsin. Suitability studies with measles and rubella viruses showed that the virus growth curve patterns as well as harvest-wise virus titres were comparable with those obtained using conventional porcine trypsin.

Conclusion

All the three trypsins were comparable with respect to cell culture suitability, effect on senescence of MRC-5 cells, Measles and Rubella Vaccine Production. Based on the results with respect to all the important parameters, recombinant trypsin proves to be the best alternative to animal origin trypsin, thereby eliminating the risk of adventitious agents entering into the manufacturing process of above viral vaccines.

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