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Multivariate Based Variability within Diverse Indian Mustard (*Brassica juncea* L.) Genotypes

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Abstract

Indian mustard (*Brassica juncea* L.) germplasm consisting 167 accessions including one check cultivar was evaluated for qualitative and quantitative traits. The present study was conducted to investigate genetic diversity and correlation among studied genotypes of *B. juncea* L. based on agro-morphological at NARC, Islamabad, Pakistan. To investigate the genetic diversity based on morphological characters, data was recorded on 20 quantitative and 12 qualitative traits. The calculated data was analyzed through two complementary methods, *i.e.* PCA (Principal Component Analysis) and cluster analysis. Among all the studied cultivars, significant diversity was recorded for different agro-morphological characters. Among all the parameters, maximum variance was recorded for pod shattering (427.2) followed by plant height (345.6), days to 100% flowering (336.2) and main raceme length (210.0). Among all the characters, the greatest and highly significant association (0.99) was found between days to maturity 50% and days to maturity 100% followed by correlation (0.86) among days to flowering 50% and days to flowering 100%, correlation value (0.71) was calculated among leaf length and leaf width. Using cluster analysis all the genotypes were divided into five major groups. It was observed that 7 out of 20 principal components with an Eigen value of ≥1.0 calculated for 73.92% of the total diversity observed between 167 accessions of Indian mustard (*B. juncea* L.). The contribution of first three PCs in the total PCs was 23.25, 12.87 and 11.24, respectively. Among all the investigated accessions two genotypes 26,813 and 26,817 showed great

potential for seeds/silique, 1000-seed weight and seed yield/plant, respectively, so these genotypes are recommended for future breeding programs for achieving promising results.

Keywords

Genetic Diversity, Agro-Morphology, *Brassica juncea* L., PCA Analysis

1. Introduction

Brassica juncea L. commonly known as Indian mustard is globally used as oil-seed, vegetable and condiments. The crop species is said to be one of the earliest domesticated species. *B. juncea* annually grows as wild and cultivated species over a large geographical range, across the Asia, Africa, Australia, America and Europe [1]. In the genus Brassicaceae, Indian mustard is one of the highly significant crop species. In Pakistan after cotton and rape seed, Indian mustard is the third highly important source of oil [2] [3].

So for the prosperity of the country, it is essential to increase the production of these crops and for maximum production, exploitation of genetic diversity of these crops is essential. Pakistan being an agricultural country but unfortunately it is deficient in the oilseed production and a large amount ($ 1054.7 million) is spent on the import about 77% of edible oil. After petroleum, Pakistan pays the second largest import bill for edible oil among food items [4].

Diversity can be determined by three different levels, first one is genetic diversity, in which the variation observed at gene and germplasm level, the second one is diversity of species, in which richness of species at a specific location and the third one is the Eco-system diversity, in which crop species interact with their environment. Diversity is potent for different crops to fulfill the gap between demand and production across the world [5]. Various morpho-biochemical and molecular methods are used to study genetic variability among local and exotic plant germplasm. The proper evaluation of important crop species helps in the identification and utilization of improved genotypes [6]-[11]. Therefore, the present study was performed to estimate and characterize the genetic variation and relationships among Indian mustard genotypes through agro-morphological traits to identify and select promising germplasm for traits of economic significance.

2. Materials and Methods

Plant material comprises 167 germplasm including 1 check cultivar of *B. juncea* L. for estimation of agro-morphological variation. The germplasm were provided by PGRI gene-bank, NARC, Islamabad, Pakistan. The present research work was conducted in the experimental area of NARC (33˚43'N and 73˚06'E) during the year 2012-2013 using augmented design. The average annual rainfall in this area ranges from 500 - 900 mm with 70% in summer and 30% in winter.

Row to row distance of 70 cm was maintained with one accession per row and the length of the row was 2.5 meter for each and every accession and genotype. During seed bed preparation the field was irrigated before sowing to provide conditions of optimum moisture while afterword no irrigations were given and the crop was grown under rain-fed conditions. The experimental germplasm were planted at a depth of 3 - 4 cm using hand drill. After germination to maintain proper plant population thinning was carried out by hand. After 30 days of germination weeding was carried out and because of no serious attack of insect pest no insecticides and pesticides were sprayed throughout the plant growth stage. The crop was harvested when almost more than 75% of plants were turned yellowish in color. Data were recorded for twenty quantitative traits such as Days to 50% flowering, Days to 100% flowering, Days to 50% maturity, Days to 100% maturity, Leaf length, Leaf width, Leaf width/leaf length ratio, Leaves per plant, Plant height, Primary branches per plant, Main raceme length, Silique per main raceme, Silique length, Silique width, Silique length and silique width ratio, Stem thickness, Seed per silique, 1000-seed weight, Seed yield per plant, Shattering percentage (%). The description of calculating different parameters is given in Table 1. The recorded morphological traits data were averaged and analyzed for simple statistics *i.e.* mean, variance, range, frequency distribution, coefficient of variance and standard deviation using computer software (MS-Excel 2007). Cluster and principal component analysis (PCA) was performed on the recorded data for quantitative traits. Before to cluster and PCA, mean of each parameter was standardized so that to avoiding scaling differences effects. For all the pairs of accessions Euclidean distance co-efficient were calculated. The Euclidean dissimilarity co-efficient matrices were utilized to estimate the association among the *B. juncea* germplasm with cluster analysis through complete linkage method (Statistica version 7.0 and NTSYS pc v 2.1).

3. Results

3.1. Correlation Study Based on Agronomic and Morphological Traits

During the present research work days to 50% flowering exhibited highly significant positive correlation with the parameter *i.e.* days to 100% flowering (0.86**), leaf length (0.28**) and leaves per plant (0.21**), similarly days to 50% flowering, had significant positive correlation with days to maturity 100% and leaf length/leaf width ratio (0.18*), plant height (0.17*), days to maturity 50%, leaf width and primary branches per plant (0.16*). While days to flowering 50% had significant negative correlation was observed with silique width (−0.18*). Days to 100% flowering had highly positive significant correlation with leaf length (0.37**), days to maturity 100% (0.29**), days to maturity 50%, leaves per plant and stem thickness (0.27**) and primary branches per plant (0.24**). While the trait like plant height (0.19*) and leaf width (0.18*) had found significant positive correlation with days to 100% flowering (Table 2).

Table 1. Detailed information for calculating 20 different quantitative traits of *B. juncea* L. genotypes.

Characters	Range	Description
Days to Flowering 50%	<80	Early
	80 - 90	Medium
	91 - 100	Average
	101 - 110	Late
	>110	Very Late
Days to Flowering 100%	<90	Early
	90 - 105	Medium
	106 - 115	Average
	116 - 130	Late
	>130	Very Late
Days to Maturity 50%	<140	Early
	140 - 150	Medium
	151 - 160	Average
	161 - 170	Late
	171 - 180	Very Late
Days to Maturity 100%	<150	Early
	150 - 165	Medium
	166 - 180	Average
	181 - 195	Late
	196 - 210	Extreme Late
Leaf Length (cm)	<5.0	Small
	5.1 - 15.0	Medium
	15.1 - 25.0	Large
	25.1 - 35.0	Extra-Large
Leaf Width (cm)	<5.0	Small
	5.1 - 10.0	Medium
	10.1 - 15.0	Average
	15.1 - 20.0	Large
Leaf Length/ Leaf Width Ratio	0.8 - 2.0	Small
	2.1 - 3.0	Medium
	3.1 - 4.0	Average
	>4.0	Large
Leaves Per Plant	9.0 - 12.0	Minimum
	12.1 - 16.0	Medium
	16.1 - 20.0	High
	20.1 - 24.0	Maximum
Stem Thickness (mm)	<15.0	Thin
	15.0 - 20.0	Average
	20.1 - 25.0	Thick
	>25.0	Highly thick

Continued

Plant Height (cm)	<120.0	Dwarf
	120.0 - 140.0	Medium
	140.1 - 160	Average
	160.1 - 180.0	Tall
	>180.0	Taller
Primary Branches Per Plant	<5.0	Minimum
	5.0 - 10.0	Medium
	10.1 - 15.0	High
	>15.0	Maximum
Main Raceme Length (cm)	<20.0	Short
	20.0 - 40.0	Medium
	40.1 - 60.0	Large
	60.0 - 80.0	Very Large
Siliquae Per Main Raceme	<20	Less
	20.0 - 40.0	Less than Average
	40.1 - 60.0	Average
	60.1 - 80.0	More than Average
	>80.0	Highest
Silique Length (mm)	<35.0	Very Short
	35.0 - 45.0	Short
	45.1 - 55.0	Average
	>55.0	Maximum
Silique Width (mm)	<4.0	Thin
	4.0 - 5.0	Greater
	>5.0	Maximum Thickness
Silique Length/Silique Width Ratio	<10.0	Small
	10.0 - 12.0	Average
	>12.0	Large
Seed Per Silique	<15.0	Minimum
	15.0 - 20.0	Average
	>20.0	Maximum
1000-Seed Weight (g)	<4.0	Minimum
	4.0 - 8.0	Average
	>8.0	Maximum
Seed Yield Per Plant (g)	<5.0	Minimum
	5.0 - 10.0	Average
	10.1 - 15.0	More than Average
	>15.0	Maximum Yield
Shattering %	<20	High Resistant
	20.0 - 40.0	Low Resistance
	40.1 - 60.0	Medium Shattering
	60.1 - 80.0	High Shattering
	>80.0	Extreme Resistance

Table 2. Correlation coefficient among agro-morphological characters in *B. juncea* L. accessions.

Trait	DF50	DF100	DM50	DM100	LL	LW	LL/LW	L/P	ST	PH	PB/P	MRL	S/MR	SL	SW	SL/SW	S/S	TSW	SY/P	SH
DF50	1																			
DF100	0.86**	1																		
DM50	0.16*	0.27**	1																	
DM100	0.18*	0.29**	0.99**	1																
LL	0.28**	0.37*	0.30**	0.31**	1															
LW	0.16*	0.18*	0.12	0.12	0.71**	1														
LL/LW	0.18*	0.22**	0.21**	0.24**	0.27**	−0.41**	1													
L/P	0.21**	0.27**	0.20**	0.19*	0.49**	0.37**	0.15*	1												
ST	0.12	0.27**	0.42**	0.43**	0.29**	0.30**	−0.1	0.41**	1											
PH	0.17*	0.19*	0.06	0.05	0.29**	0.47**	−0.33**	0.36**	0.45**	1										
PB/P	0.16*	0.24**	0.30v	0.28**	0.44**	0.50**	−0.1	0.41**	0.43**	0.46**	1									
MRL	0.02	0.05	0.08	0.05	0.15*	0.18*	−0.17*	0.20**	0.14	0.38**	0.26**	1								
S/MR	0.11	0.14	0.40**	0.38**	0.40**	0.27**	0.05	0.30**	0.22**	0.32**	0.31**	0.59**	1							
SL	−0.06	−0.08	0.14	0.16*	−0.04	−0.08	0.03	−0.13	−0.09	−0.19*	0.07	−0.08	−0.09	1						
SW	−0.18*	−0.12	0.18*	0.19*	0.05	0.02	0.02	0.1	0.08	−0.1	0.06	0.08	0.15	0.16*	1					
SL/SW	0.13	0.05	−0.08	−0.08	−0.08	−0.07	−0	−0.18*	−0.12	−0.03	0	−0.12	−0.18*	0.50**	−0.75**	1				
S/S	−0.06	−0.07	0.29**	0.30**	0.20**	0.04	0.23**	0.03	−0.1	−0.14	0.01	−0.04	0.18*	0.02	0.26**	−0.25**	1			
TSW	0.03	0	0.18*	0.18*	0.03	−0.02	0.1	0	0.03	0.03	0.07	0.15	0.22**	−0.06	0.19*	−0.21**	0.16**	1		
SY/P	0.07	0.11	0.42**	0.41**	−0.02	−0.11	0.15*	0.1	0.20**	0	0.09	−0.07	0.05	0.08	0.06	0.02	0.1	0.06	1	
SH	−0.02	−0.08	−0.35**	−0.37**	−0.32**	−0.18*	−0.18*	−0.26**	−0.12	−0.17*	−0.21**	−0.23**	−0.43**	0	−0.1	0.09	−0.31**	−0.1	−0	1

Note: *significant at 0.05 (0.15 to 0.19 value); **highly significant at 0.20 & above probability level (more than 0.00 level); The green, yellow, white, red colors shows strong positive, weak positive, moderate positive, and strong negative correlations among different traits respectively.

3.2. Principal Component Analysis Based on Agronomic and Morphological Traits

Principal component analysis (PCA) was carried out based on twenty quantitative morphological characters. The 7 principal components account for 73.92% of the overall variability among the studied *B. juncea* L. accessions for the total phenotypic variations (Table 3, Figures 1-6). Among these seven principal components (PCs), the PCA-I was found to have 23.35% out of the total variability. Silique length (0.073), silique length/width ratio (0.224) and shattering percentage (0.506) contributed positively to first principal component. In contrast, days to flowering 50% and 100% (−0.385 and −0.494), days to maturity 50% and 100% (−0.680 and −0.679), leaf length (−0.711), leaf width (−0.569), leaf length/leaf width ratio (−0.112), leaves per plant (−0.616), stem thickness (−0.606), plant height (−0.513), primary branches per plant (−0.645), main raceme length (−0.391), siliqua per main raceme (−0.653), silique width (−0.193), seeds per silique (−0.230), 1000-seed weight (−0.203) and seed yield per plant (−0.251) contributed negatively.

The contribution of PC-II in total differences was 12.87% and was positively

associated with days to maturity 50 and 100% (0.520 and 0.540), leaf length/width ratio (0.540), silique on main raceme (0.034), silique length (0.234), silique width (0.435), seed per silique (0.530), 1000-seed weight (0.270) and seed yield per plant (0.394), whereas days to flowering 50 and 100% (−0.176 and −0.133), leaf length (−0.149), leaf width (−0.506), leaves per plant (−0.187), stem thickness (−0.153), plant height (−0.580), primary branches per plant (−0.280), main raceme length (−0.243), silique length/width ratio (−0.245) and shattering percentage (−0.215) were negative associated with PC-II.

PC-III showed 11.24% of the total agro-morphological variation and positively associated with leaf width (0.161), leaves per plant (0.033), plant height (0.144), primary branches per plant (−0.015), main raceme length (0.365), siliqua per main raceme (0.266), seeds per silique (0.205), 1000-seed weight (0.232) and silique width (0.578), while negatively associated with days to flowering 50%

Table 3. Principal components (PCs) of agronomic traits among *B. juncea* L. genotypes.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigen value	4.67	2.57	2.25	1.61	1.38	1.27	1.03
Cumulative Eigen value	4.67	7.24	9.49	11.11	12.48	13.75	14.78
Percent Variance	23.35	12.87	11.24	8.07	6.89	6.35	5.15
Cumulative Variance	23.35	36.22	47.46	55.54	62.42	68.77	73.92
Traits	**Eigenvectors**						
Days to Flowering (50%)	−0.385	−0.176	−0.610	−0.463	−0.054	0.138	−0.309
Days to Flowering (100%)	−0.494	−0.133	−0.587	−0.443	−0.135	0.095	−0.251
Days to Maturity (50%)	−0.680	0.520	−0.183	0.273	−0.151	0.063	0.022
Days to Maturity (100%)	−0.679	0.540	−0.207	0.252	−0.147	0.037	0.016
Leaf Length	−0.711	−0.149	−0.066	−0.186	0.329	−0.396	0.004
Leaf Width	−0.569	−0.506	0.161	0.093	0.103	−0.417	−0.107
Leaf Length/Width Ratio	−0.112	0.540	−0.353	−0.423	0.274	−0.021	0.192
Leaves/Plant	−0.616	−0.187	0.033	−0.180	−0.026	−0.196	0.228
Stem Thickness	−0.606	−0.153	−0.029	0.193	−0.492	−0.025	0.165
Plant Height	−0.513	−0.580	0.144	0.133	−0.118	0.155	0.106
Primary Branches/Plant	−0.645	−0.280	0.015	0.243	−0.033	−0.160	−0.116
Main Raceme Length	−0.391	−0.243	0.365	0.095	0.246	0.563	−0.143
Siliqua/Main Raceme	−0.653	0.034	0.266	0.040	0.319	0.394	−0.030
Silique Length	0.073	0.234	−0.290	0.592	0.228	−0.224	−0.509
Silique Width	−0.193	0.435	0.578	−0.108	−0.253	−0.259	−0.414
Silique Length/Width Ratio	0.224	−0.245	−0.696	0.490	0.349	0.090	0.022
Seeds/Silique	−0.230	0.530	0.205	−0.103	0.332	−0.238	0.059
1000-Seed Weight	−0.203	0.270	0.232	−0.116	−0.007	0.378	−0.338
Seed Yield/Plant	−0.251	0.394	−0.248	0.236	−0.388	0.125	0.248
Shattering (%)	0.506	−0.215	−0.119	−0.069	−0.445	−0.069	−0.295

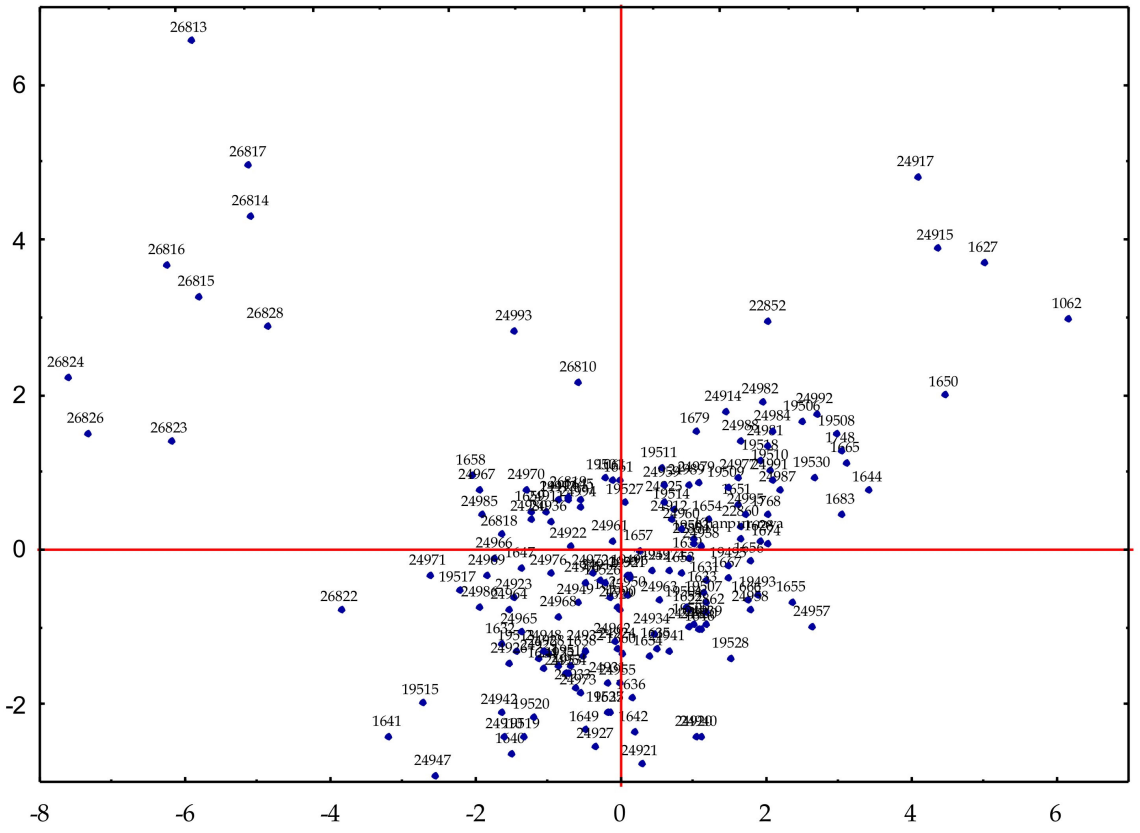

Figure 1. Scatter diagram og PC-I and II for agro-morphological traits.

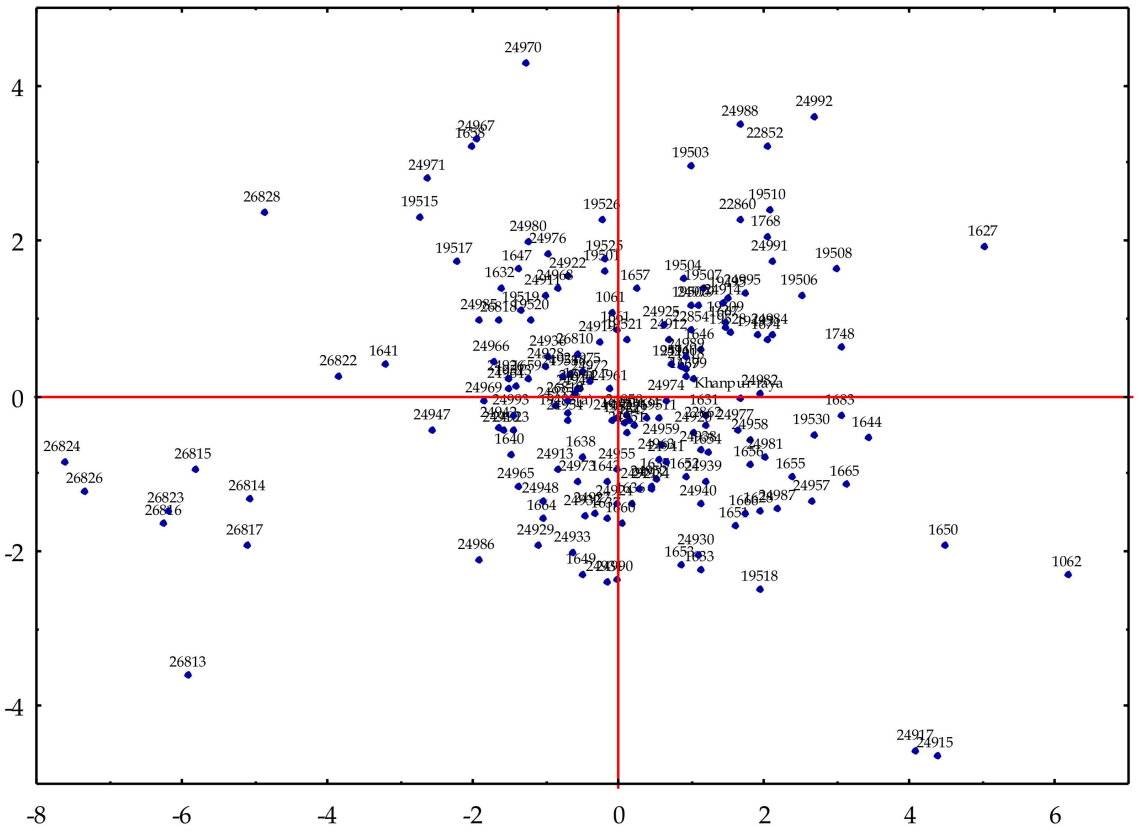

Figure 2. Scatter diagram of PC-I and III for agro-morphological traits.

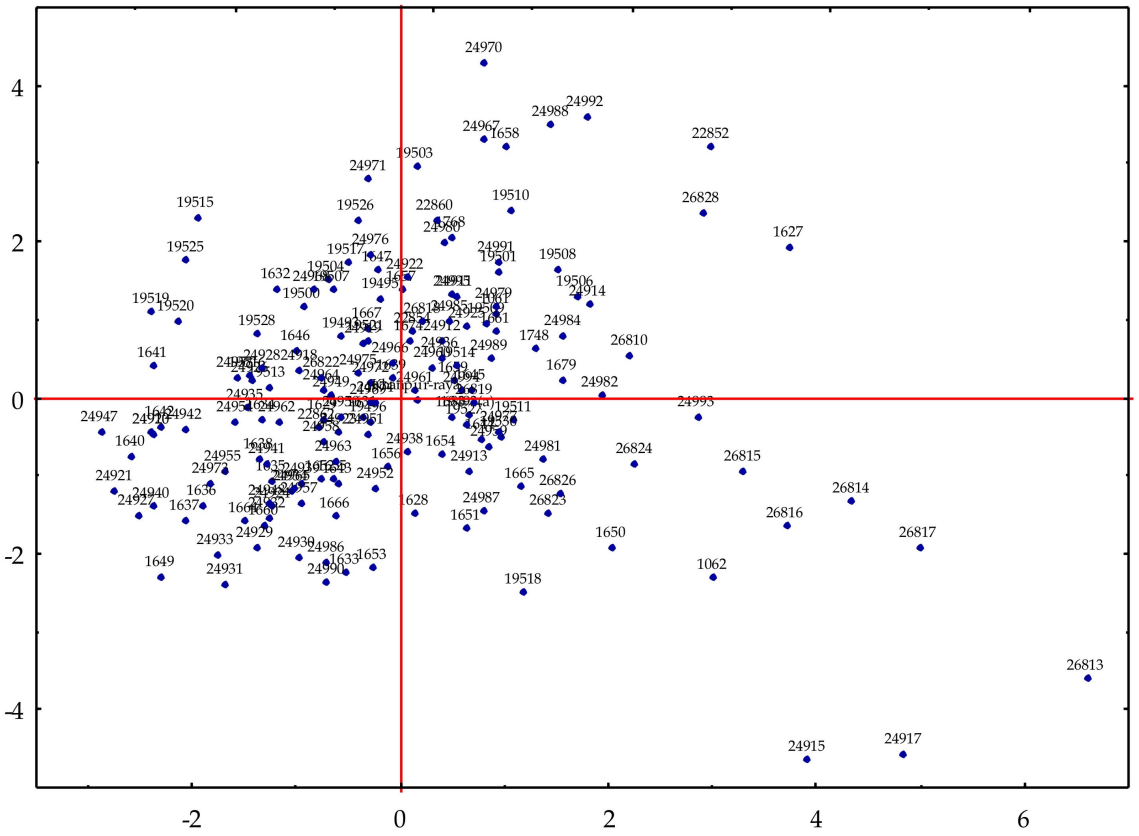

Figure 3. Scatter diagram of PC-II and III for agro-morphological traits.

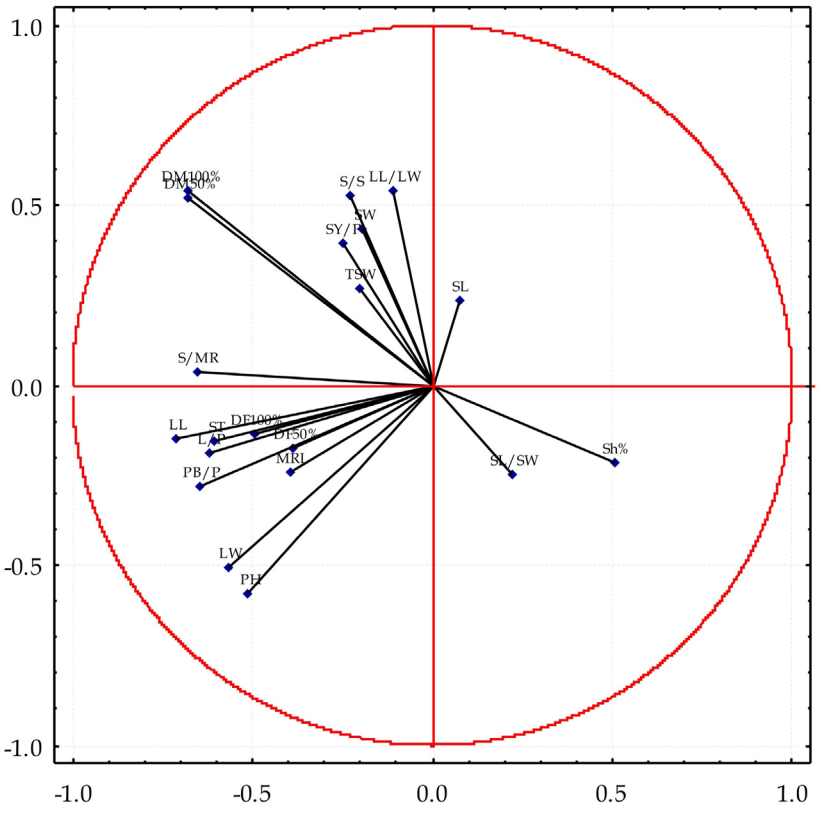

Figure 4. Contribution of morphological traits in the first two principal components.

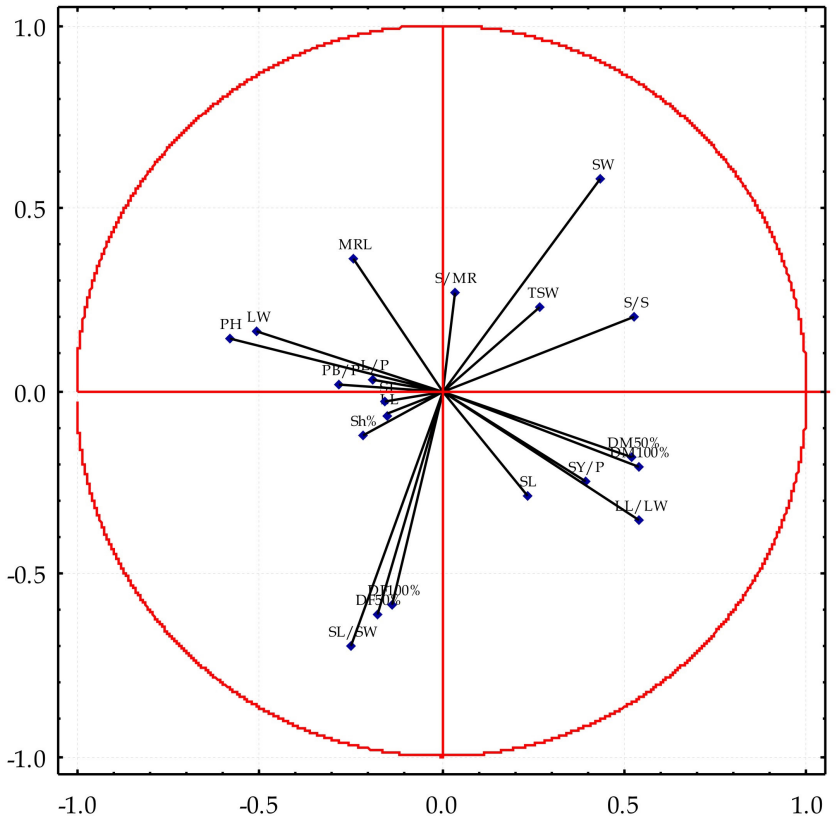

Figure 5. Contribution of morphological traits in the second and third principal components.

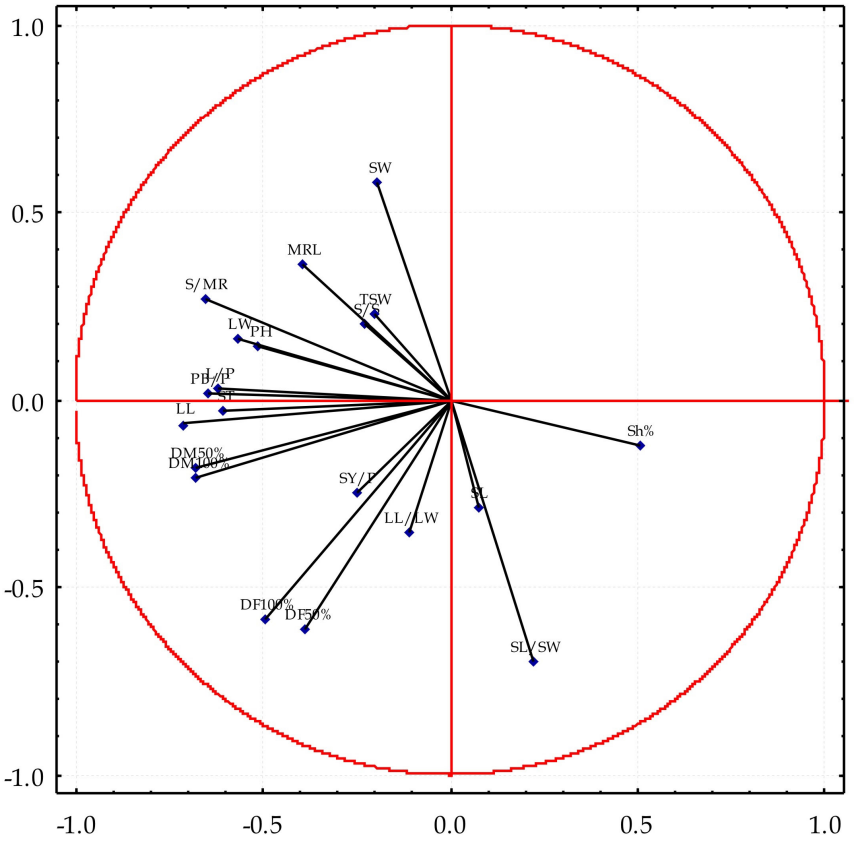

Figure 6. Contribution of morphological traits in the first and third principal components.

and 100% (−0.610 and −0.587), days to maturity 50% and 100% (−0.183 and −0.207), leaf length (−0.066), leaf length/leaf width ratio (−0.353), stem thickness (−0.029), silique length (−0.290), silique length/width ratio (−0.696), seed yield per plant (−0.248) and shattering percentage (−0.119).

The PC-IV contributed 8.07% in the total variability and were positively contributed to days to maturity 50% and 100% (0.273 and 0.252), leaf width (0.093), stem thickness (0.193), plant height (0.133), primary branches per plant (0.243), main raceme length (0.095), silique per main raceme (0.040), silique length (0.592), silique length/width ratio (0.490) and seed yield per plant (0.236). Similarly the traits *i.e.* days to flowering 50% and 100% (−0.463 and −0.443), leaf length (−0.186), leaf length/width ratio (−0.423), leaves per plant (−0.180), silique width (−0.108), seed per silique (−0.103), 1000-seed weight (−0.116) and shattering percentage (−0.069) contributed negatively.

In the total variability the contribution of PC-V was observed 6.89% and the parameter *i.e.* leaf length (0.329), leaf width (0.103), leaf length/width ratio (0.274), main raceme length (0.246), siliqua per main raceme (0.319), silique length (0.228), silique length/width ratio (0.349), seeds per silique (0.332) were positively contributed. Whereas the characters *i.e.* days to flowering 50 and 100% (−0.054 and −0.135), days to maturity 50% and 100% (−0.151 and −0.147), leaves per plant (−0.026), stem thickness (−0.492), plant height (−0.118), primary branches per plant (−0.033), silique width (−0.253), 1000-seed weight (−0.007), seed yield per plant and shattering percentage (−0.388 and −0.445) contributed negatively.

The PC-VI depicted proportion of variability as 6.35% and were positively associated with days to flowering 50% and 100% (0.138 and 0.095), days to maturity 50 and 100% (0.063 and 0.037), plant height (0.155), main raceme length (0.563), siliqua per main raceme (0.394), silique length/width ratio (0.090), 1000-seed weight (0.378), and seed yield per plant (0.125). Similarly the traits *i.e.* leaf length (−0.396), leaf width (−0.417), leaf length/width ratio (−0.021), leaves per plant (−0.196), stem thickness (−0.025), primary branches per plant (−0.160), silique length (−0.224), silique width (−0.259), seeds per silique (−0.238) and shattering percentage (−0.069) were negatively associated with PC-VI.

PC-VII accounted 5.15% of the total variation among agro-morphological traits. Days to maturity 50% and 100% (0.022 and 0.016), leaf length (0.004), leaf length/width ratio (0.192), leaves per plant (0.228), stem thickness (0.165), plant height (0.106), silique length/width ratio (0.022), seeds per silique (0.059) and seed yield per plant (0.248) were contributed positively to PC-VII. Whereas days to flowering 50% and 100% (−0.309 and −0.251), leaf width (−0.107), primary branches per plant (−0.116), main raceme length (−0.143), siliqua per main raceme length (−0.030), silique length (−0.509), silique width (−0.414), 1000-seed weight (−0.338) and shattering percentage (−0.295) depicted negative contribution.

4. Discussion

Maximum variation was calculated for shattering (427.2) followed by plant

height (345.6), days to flowering 100% (336.2), main raceme length (210.0), days to flowering 50% (185.1), days to maturity 100% (122.9), days to maturity 50% (83.8). The results obtained from the present investigation were in closed agreement with that of [12] [13] [14] [15].

Our results were strengthened by many earlier plant breeders and scientists. The highly significant and positive correlation was observed between the parameters of days to flowering 50% and 100% and days to maturity 50% and 100% physiological maturity. These results were supported by the results of Nasim *et al.* [13], Alemayehu and Becker [16], by reporting highly significant and positive correlation among days to 50% and 100% flowering and days to 50% and 100% maturity. The results obtained by Zare [17] were also strongly supported our finding, who reported positive and highly significant correlation among important traits like days to flowering, days to maturity and plant height of *B. napus* L. accessions. Similarly Azadgoleh *et al.* [18] also reported positive and highly significant association among days to physiological maturity and plant height. Similarly the results of Rabbani *et al.* [19] also strengthen our findings. In the current investigation main raceme length was highly significant positive correlated with the trait of number of silique per main raceme. The results obtained by Rabbani *et al.* [19] and Nasim *et al.* [13] had also confirmed our findings. Plant height was also found positive and highly significant correlated to primary branches per plant, main raceme length and silique per main raceme which was also close agreement by previous research of Yasir *et al.* [20] who studied strong positive and significant association of plant height with primary branches per plant, main raceme length, pod on main raceme, pod length and seeds per pod.

Numerous plant breeders and plant scientist in the earlier era had practiced and got remarkable results of diversity in agro-morphological parameters for various Brassica individuals through two complementary methods *i.e.* cluster analysis and principal component analysis (PCA). These techniques were successfully observed in Indian mustard (*Brassica juncea* L.) germplasm by Gupta *et al.* [21], in Ethiopian mustard by Alemayehu and Becker [16] and in white head cabbage Balkaya *et al.* [22]. All the above mentioned plant breeders and plant scientist's results gives support to our present investigation that these 2 techniques are very supportive in estimated associations among individuals originated from different environments in a more clear approach. The classification of present studied *Brassica juncea* L. genotypes into various groups was not due to of its origination from diverse ecological zones of the country and world. Amurrio *et al.* [23], who investigated qualitative and quantitative traits of Iberian pea genotypes, were agreement with our results. During the present investigation among different accessions of *Brassica juncea* L. considerable level of genetic differences was observed for different agronomic and morphological characters. These studied parameters can play significant and interesting role in *Brassica juncea* L. cultivars and varieties development programs in future. In present study the seven groups were recorded and PC1 showed highest level of variability. The same findings were also recorded by Dias *et al.* [24] in diverse

germplasm of *Brassica oleracea* L. originated from Portugal.

5. Conclusion

Among all the characters, valuable genetic diversity was observed through different agro-morphological traits *i.e.* days to maturity 50% and days to maturity 100%, days to 50% flowering and days to flowering 100%, leaf length and leaf width, main raceme length siliquae per main raceme, leaf width, primary branches per plant, silique length. Most of the traits contributed highly significant association for the said trait during the present investigation. These genotypes show that there is great potential in the studied accessions, which can be used in future breeding program as a productive genotypes.

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Conflict of Interest

The author's declares that there is no conflict of interests regarding the publication of this article.

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Single Chain Fragment Variables Antibody binding to EGF Receptor in the Surface of MCF7 Breast Cancer Cell Line: Application and Production Review

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Abstract

In this review, single-chain fragment variable construction using phage-display technology as a promising anticancer immunotherapy technology is described. Cloning and the specific bio-panning selection with phage display technology, as well as the use of the epidermal growth factor receptor (EGFR) at the surface of MCF-7 cells as the antigen for the straightforward specific selection of single chain Fvs, are discussed. Moreover, phage display technologies and their application are important for vaccine production and immunotherapy against viruses and cancers. Furthermore, expression of the gene will cause the production and expression of the protein in prokaryotic and eukaryotic cells, which can be used to detect anti-cancer single chain fragment variables (scFvs). Finally, homology modelling is described to show the three-dimensional scFv structure that verifies the Complementary-Determining-Regions (CDRs) on the surface of the model.

Keywords

Single Chain Fragment Variable, Epidermal Growth Factor Receptor, MCF-7, Phage Display Technology

1. Introduction

Breast cancer is the second major reason of death in women in age between 40 and 55 years after lung-cancer. Breast cancer is caused by the abnormal growth of cells in the mammary gland [1]. Moreover, the molecular conversion of the breast tissue from normal to malignant indicates that breast cancer is a genetic disease, subsequent from series of genetic lesions. Because there are restrictions

to the current treatment routines for this genetic disease, novel therapeutic modalities will be beneficial for the effective treatment of breast cancer. Sum of gene therapy methods for breast cancer have been advanced. These methods can be alienated into the following six mean classes: 1) genetic assimilation therapy; 2) chemo-therapy; 3) disintegrate cell therapy; 4) gene therapy to reduce the growth of new blood vessels tumors that need to grow; 5) genetic immune-resistance; and 6) genetic resistance therapy. Those clinical trials indorsed the security and possibility of these different gene therapy methods for breast cancer. Gene therapy can be established by using malignant-targeted monoclonal antibodies (Mabs) alone or Mabs bind to radio-substances, drugs, which kill cancer cells while leaving normal cells unhurt.

The use of IgG monoclonal antibodies is first start antibodies produced in hybridoma technology. Those antibody targeted EGFR on the surface of MCF-7 breast cancer cells, which is function in to gene therapy and diagnosis of MCF-7 cancer cell. The growth-factor-receptors play significant parts in controlling cell propagation, that directed the attention into use them as a drug for cancer therapy. Consequently, growth-factor-receptors are best applicants for genetic therapy because they are normally over-expressed on the surface of malignant cells. Additionally, [2] described the important role growth-factors could play to diagnosis malignant cells, demonstrating that tyrosine kinase (TKGFR 1) growth-factor-receptor is produced in a wide variety of malignant cells that categorises them as unique complicated cell-surface indicators for human tumours.

Single chain fragment variables are molecular formed of IgG monoclonal antibodies. Which are constructed using phage display technology and molecular cloning and the specific bio-panning selection with the use of the epidermal growth factor receptor (EGFR) at the surface of MCF-7 cells as the antigen for the straightforward specific selection are considered as fast and accurate way of producing specific antibodies [3]. In this review, the process of constructing a single chain antibody by using phage display technologies, which are sequence of known experiments, that in the end produce high specific antibodies described in detail. The scFv-MCF-7 products are highly recommended to be used in diagnosis and gene therapy toward breast cancer cell line MCF-7.

2. Anti-EGFRvIII MCF-7 Monoclonal Antibodies

There various Mabs directed specifically towards EGFRvIII of MCF-7 with great-affinity that are produced using hybridoma technology [4]. These EGFRvIII-bound specifically with monoclonal antibodies have been produced to measure the activity and amount of the EGFRvIII protein expressed in surgically removed tissue samples that collected from human breast carcinoma. MAbs and sc-Fv manufactured specific for the mutant EGFRvIII have been developed, even though EGFRvIII does not bind EGF, these ligand-induced pathways are unrelated and are less specific to reach their aims. However, the future of an anti-EGFRvIII Mab and scFvs after binding to EGFRvIII remains uncertain. Based on immune-fluoresces-imaging and radio-EGFRvIII-labelled Mab assays, clear-

ly, the intracellular vesicles are effected in amount of mints when, specific connection of Mabs with EGFRvIII took place [5]. Reist [5] demonstrated the expression of a human IgG_2/mouse chimeric anti-EGFRvIII Mab, and the significant characteristics of the chimeric Mab are comparable to the murine parent. still, the chimeric construct showed more significant specificity in comparative tissue distribution studies of 8.2% in compared with 13.4% to the parent Mabs with P = 0.0009 towards targeting receptors *in vivo*. Production of MR1, and its affinity-developed generation-1 MR1 [2], are great future therapeutics candidates.

3. General Structures of Antibodies

In human natural immunity, there are similar group of glycol-proteins that stimulate immunity response in humans [6]. Five classes (isotypes) of immune-globulins Ig (G, A, M, D, and E) have been recognised, each of them has a separated heavy-chain C region programmed by a separated C-region gene. The isotype of an immune-globulin controls the human body immune-process that it involves during antigen binding. Each domain describes the characteristic immune-globulin fold containing of two beta-sheets anti-parallel with disulphide bond. The varied choice of specificities on the surface of Mabs performed by number of amino acids consists of six hyper-variable loops or complementarity-determining-regions (CDRs), which formulate the epitopes. Those CDRs, maintained specific frame-work-region, establish the changeable regions of fragment antigen binding (Fab). The enduring V section amino acids act as a scaffold supporter of the loops and are known as framework residues as demonstrated by [7].

The Fc fragment interacts with effector systems, such as the complement system, as shown in **Figure 1**. The two domains of light chain (L) is consist of 100 residues in length, called the variable-light (VL) and the constant (CL) domain as shown in **Figure 2**. The heavy chain (H) consist of variable-domain (VH) and the four constant C (H1, H2, H3, and H4) domains, as described [8]. Each domain contains two beta sheets filled facing each other and linked together by a well-maintained disulphide bridge and inter-strand loops as described by Lesk and Chothia [9]. Disulphide bridges are an important component of the IgG structure. The disulphide bridge stabilises domain folding, and disulphide bridge stabilise the interaction between H chain, L chain and the interaction of H chains [10].

4. The Variable Domain Regions

Ab contacts with the antigen are helped by the variable domains V (H and L) as shown in **Figure 1**. Most of antibody-antigen interactions performed by the complementarity determining regions (CDRs). Regarding to the size of the antigen, different mixtures of the CDR loops are used for the contact with antigen [11]. Commonly, antibodies directed towards small antigens, such as viruses, the antibody use three complementarities determining regions. However, antibodies

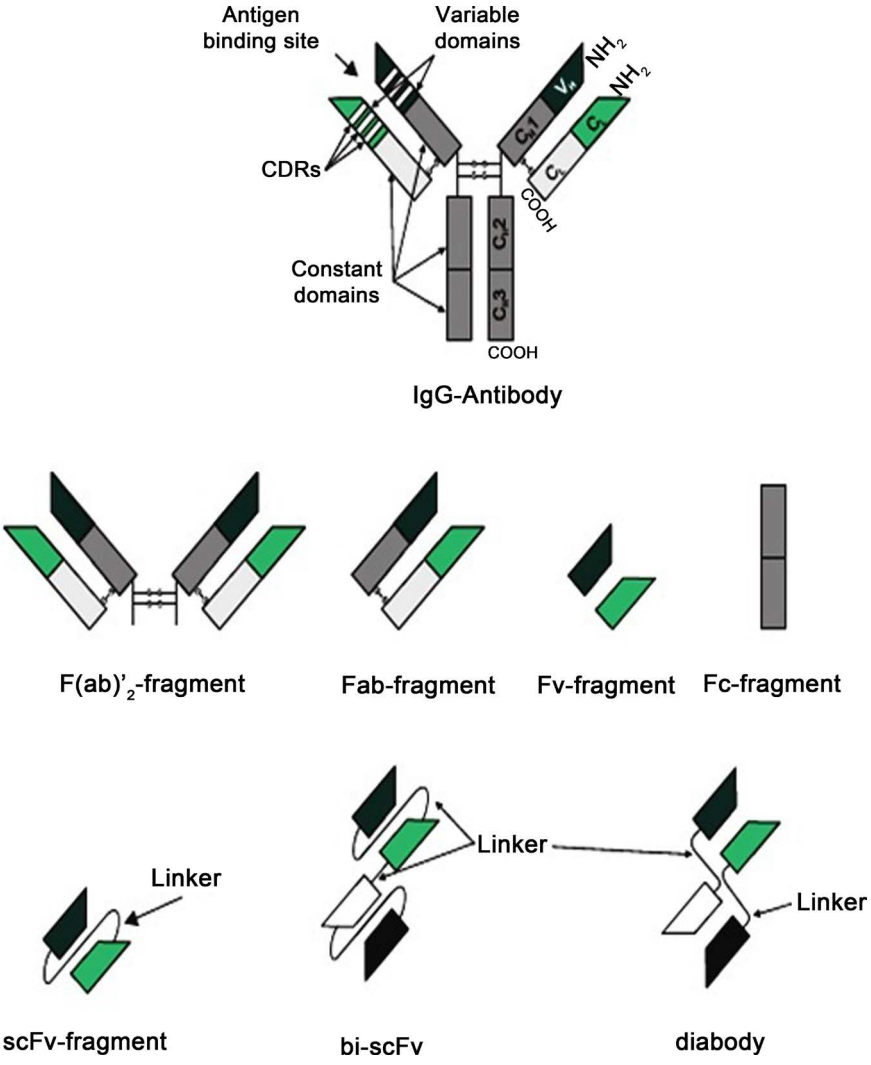

Figure 1. Schematic presentation of IgG molecule and antibody fragments [35].

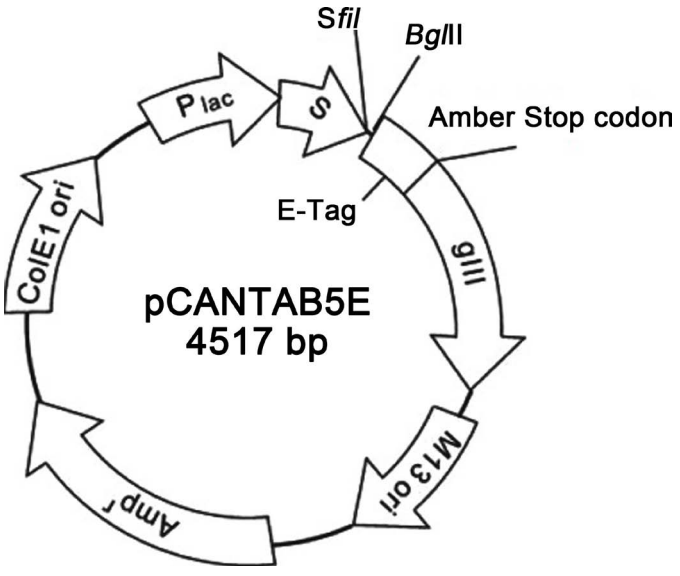

Figure 2. Phage display vector pCANTAB 5E. The restriction sites *SfiI* and *NotI* are used to insert interest gene. Arrows indicate functional direction of genes [44].

directed towards larger proteins use more than four CDRs.

MAbs are working mostly helped by their fragment chain (Fc), collected of domains C (H2 and H3). Still, antibody-dependent cellular cytotoxicity and Ab-dependent cellular phagocytosis ((ADCC and ADCP) in humans demonstrated the finding of three major classes of Fc FcλRI, FcλRIIa/c, Fcλ RIIIa, and inhibitory FcλRIIb receptors [12]. The FcR and Fc-RI receptors of extracellular regions in humans show significant sequence similarity of 70% - 98%. The antibody binding to the components of the complement system is activated with Complement dependent cytotoxicity (CDC). In addition, the antibodies get their long half-life through active recycling of the neonatal Fc receptor [13].

Scfv is expressed in a variety of hosts, including bacteria, yeast and plants [14]. Single-chain fragments have broad applications in medicine and have great potential in biotechnology. The unique and highly specific antigen-binding ability may be exploited to block specific enzymes or bacteria or to detect environmental pollutants present in very low concentrations (biosensors) [15].

5. Immunoglobulin Genetics

Immunoglobulin (Ig) heavy chain variable genes are composed of variable-diversity-joining (VH-D-JH) gene sequences. An efficient VH-D-JH compound is shaped when B-cell initiated and VH gene sequence are linked with a D and JH gene sequences [16]. In humans, there are a 100 to 200 VH gene sequences, more than 30 D sequences, and six efficient JH gene sequences. Germ-line VH sequences are divided into six groups, VH1 to 6, based on DNA sequence similarity. The same VH groups are naturally more than 80% homologous. Still, less than 70% homology between VH gene sequences and other groups sequences. Separated VH groups differ in size from 1 to more than 28 members and composed of non-functional and functional genes. The frequency with which individual VH families are used by adult peripheral blood lymphocytes (PBL) has been reported to roughly correlate with estimates of family size based on Southern blot analysis [17].

Moreover, VH and VL sequences are amplified to build recombinant single chain F antibody fragments. The peptide linker between VH and VL sequences that manufactured single chain fragment variable. The linker contains two amino acids sequences Glycine (GlY) and serene (Ser) with repeats as GlY_4 and Ser_3. The linker with the flexible disulphide bonds, moves between the C-terminal of the VH chain and the N-terminal of the VL chain [18]. The stability, solubility and affinity are primarily influenced by the disulphide bond linkers in these molecules [19]. To construct the VH and VL genes, mRNA is isolate from a hybridoma, lymph cells or bone morrow. Then, the mRNA transcribed into DNA using reverse transcribe technology and antibody genes sequences template are then amplified by PCR. This technology requires oligonucleotide primers that can read most of antibodies sequences. This procedure has led to make large libraries that consist a wide range of antibody VH and VL gene sequence [20].

Finally, the main advantage of using small fragment as antibody in experi-

mental animals' applications, is the easiness of the small fragment to enter the organ and fast clearance from tissues and blood stream [21]. Recombinant single chain fragments have connected with wide variety of particles, such as enzymes for pro-drug treatment, toxins for cancer treatment, viruses for gene therapy, and ligands for guiding to specific cellular targets [22].

6. Application of scFv Antibodies

6.1. ScFv Antibodies as a Neutralising Agent

ScFv antibody is neutralising antibodies, which, they can connect with toxins and viruses and terminate them with high affinity. Additionally, scFv antibodies are smaller than parent antibodies that allows faster distribution in tissues and enhanced clearance of immuno-complexes from the blood stream. There are numbers of such cases in literature. For example, the scFv directed against F glycol-protein of respiratory-syncytial-virus, that decrease viral concentrations in the mice lungs, rabies-neutralising single chain fragment variable [23]. Moreover**,** wide range of neutralising single chain fragment attached to human cyto-megalo-virus glycol-proteins, isolated from a library of phage display. Furthermore, the Fab form of scFv has effective neutralising activities. The single chain fragments variable directed towards the hyper-variable loops of the Hepatitis-C-virus neutralising the C virus [24].

6.2. ScFv Antibodies in Tumours Labelling and Bio-Therapeutics

The scFv antibodies labelled with radioisotope can be used to detect tumours in vivo. The ideal candidate tumours label that would allow faster distribution in cancer tissues and enhanced clearance from the blood stream, in the result has less contact of the healthy tissue [25].

The advantage using of scFv antibody in tumours labelling is clear, for example when scFv antibody is fused to technetium-99 m [26]. Technetium is commonly used since it has short half-life of six-hour. This radiolabel agent is compatible with scFv antibody as carrier due to scFv antibody faster distribution in tissues and enhanced clearance of immuno-complexes from the blood stream. Moreover, scFvs or Fabs have worked successfully better, when they form complex molecule (multivalent) such as dimers, trimers, and tetramers. These complexes sequences of antibodies have advanced efficient affinity and were tried in many *in vivo* studies. In an earlier reported, a successful use of scFv antibody fused to chimeric-embryonic antigen (CEA) format resulted in rapid localisation to the tumour site, rapid clearance from the circulation and produced clear images.

In tumour treatment, immunotoxins are manufactured using antibodies connected to wide range of toxins, also the advantage of significant specificity of scFv antibodies in which they are connecting with cancers cells receptors with great affinity. ScFv antibodies fused to anti-cancer in various form for example cyto-toxic fragments, radio-nuclides or drugs. Gunter [27] showed that planed cancer treatment of human patient with specific scFv antibodies can handle highest drug treatment amount of 12.5 μg/kg. The tumour cells are terminated,

when scFv antibody fused to cyto-toxins such as IL2 interlukin2 is attached to their receptors [28].

6.3. ScFv Antibodies in Cancer Identification

The enormous use of scFv antibodies in identify cancer cell due to the antibodies specificity is demonstrated. The antibody can connect small fragment of the antigen or to small part of the cancer cell receptor. The scFv antibody normally constructed with tags fused to gene sequence, which they use to connect to the antibody conjugates. There are many tags in the market to be used for example: His-tags (Invitrogen) and S-tag (Merk). Still, scFv antibody face a problem of un-folding and non-soluble, when used in ELISA test, that because of its small less number of domains. To evade such problem, scFvs antibodies panned from phage display libraries can used in ELISAs, which scFv connected to the phage's coat. This could increase activity of scFv protein since the phage can replace the heavy and light sequences [29].

In other hand, constructed scFv fused with short tag sequences give the resulted protein extra activity and stability. There are many of these tags for example a constant light chain (CL), and alkaline phosphatase (AP) [29]. Furthermore, scFvs in two and three repeated complexes dimers for example divalent scFv or trivalent scFv, which gain better solubility and activity in protein 3D structure compared with original monovalent scFv [30]). Finally, scFv-AP structure, won more advantage of direct connection with substrate without the need of conjugates.

7. Phage-Display Technologies

Phage display system is a technology that constructed gene and its fusion protein are connected, that gave wide range of molecular libraries selection, using antigen-specific binding and that become possible per excessive rounds of binding and washing between the target-specific and their antibodies in a procedure called bio-panning. The most popular surface was used to display is the M13KO7 bacterio-phage, described by Smith [29].

7.1. The Phage-Mid Vector

The presentation of target-antibody on the surface of M13KO7 filamentous phage become possible with the help of phage display vectors as demonstrated by smith [29]. First, phage vectors keep all DNA information essential for the phage life-cycle [31]. The phage-mid is shorter sequence of M13KO7 used to display the antibodies in this technology [32]. The phage-mid sequence has M13KO7 middle area with its source of copying for virus strand combination, also, the hair-pin filler signal. The phage-mid also contains a source for vector copying and an antibiotics resistance.

This technology became accessible with the inventing of DNA plasmids that used selecting of target-specific antibody connected to M13KO7 phage surface in the pool of selected specific libraries. The connection of M13KO7 to the C-end

of a DNA templates sequence has stop codon which, gained after special forward and reverse primers always contain translation stop codons and to avoid transcription and eventually expression of coat proteins in the beginning of connected plasmid. In M13KO7, phage-coat short amino acids sequence such as pIII and pVIII lead their forwards primers be transcript as the basic template sequence, whereas, the role of their c-end is necessary for effective phage expression as shown in Figure 2.

7.2. Bio-Panning

It is well known that bio-panning is greatly affected by the way the phage libraries coated to expose the target antigens. The coating of Target antigen is normally on immobilise magnetic surface such as ELISA magnetic wells. The specific phage receptors connected to the target antigen, and the unconnected phage is drained away. The targeted connected phage is then collected by disturbed the contacts among the phage sequence and immobilised target antigens. The improved phage is then transferred into bacteria host to grow in large scale, *E. coli* is one of bacterial hosts, to improve phage properties as shown in Figure 3. The bio-panning procedure could be done many times, the resulted phage-antibody fused would be accordingly gained significant specificity toward the selected target antigens. After many coating and washing process, the gene sequences from strongest phage reading should be DNA sequenced to match the result with the published gene bank libraries [33].

The most import result from bio-panning amplification and washing procedures in phage-display is the wide range of selected numbers of target specific phage-antibody. The constructed target specific phage-antibody selected numbers can be more than 10^{10} [34]. The choice of selecting phage display is differ from tissue culture to sterilized mice. In tissue culture selection allows phage-antibody fusion to be coated against a various number of target antigens [35].

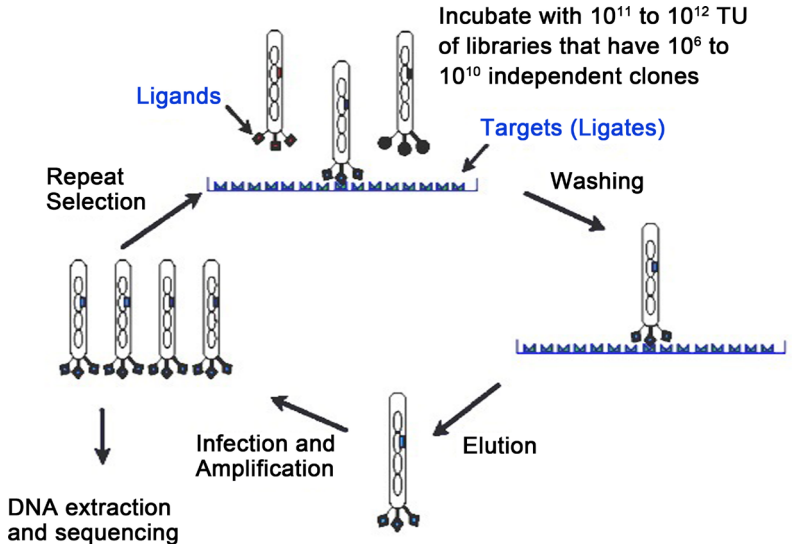

Figure 3. Bio-panning is infection of bacteria with a filamentous helper phage and some incubation steps [41].

When Yeast cell are used as target antigen, the exchanges between the fusion phage-antibody should be under specific functional environment; still, those conditions are difficult to apply in this technology, mainly in yeast cells [36].

In other hand, phage-display selecting arrangements can be easily improved to work the screening environments and inflexibilities [37]. Both the yeast-hybrid technologies and phage-display deliver a system capable of easy selection of specific phage-antibody in enamours scale directed toward certain targets, even though, phage-display has more quantity in favour. However, yeast hybrids could reach those enormous clones number in longer time compare to phage-display, whereas their clones would be selected in millions in not more than a week, whereas with yeast two-hybrids, millions of clones can be screened in two to four weeks [38].

7.3. Phage-Antibody Fusion DNA Sequences

Phage-antibody fusion could be constructed with two different methods. First phage-antibody fusion constructed from spleen mRNA of immunised donors, the other type is derived from non-immunised donors. With the first library, the phage antibody repertoire is developed for antigen-specific antibodies because some have endured immune response from natural immune system [39]. The spleen mRNA of immunised donors' yields stronger specification of phage-antibody than the one constructed from hybridoma [40]. For phage-antibody fusion pools, it is better to use a spleen sample with a higher serum antibody concentration of the target. Even though specific antibodies reflect higher levels of specific mRNA as shown in **Figure 3**.

The manufacturing of phage-antibodies pool is diversity of species of animal such as human, mice and rates has been demonstrated by Hoogenboom [41]. Moreover, the specificity of heavy and light chain fragments connected with linker directed towards target antigen is higher. However, phage-display libraries of single-library consider less specific and much low affinity. Therefore, count on the cause of the monoclonal antibodies DNA sequence is further differentiated in resulted antibody pools. The naïve pool of antibodies gives better specificity of phage-antibodies without the use for immunisation. V-sequence are constructed from B-cell cDNA template with VL and VH family primers as shown in **Figure 4** [42]. The amplified heavy and light chains are linked together and amplified to produce an advanced pool of phage-scFv antibodies. This technology offers manufacturing to antibodies that did not directed towards antigen, while the number of those open "original antibodies" is dependent on B-cells source of construction. A huge pool of libraries can be needed to produce phage-antibodies fusion to many targets proteins, such as self, non-immunogenic and quite dangerous targeted protein [43].

The immature pool of antibody shall be enormous to contain almost the whole immune collection and permit isolation of target protein receptor with strong reaction as shown in **Figure 4**. The pool of immature pool of antibodies could be million or more, still most of it is hardly recover [44]. The reading of

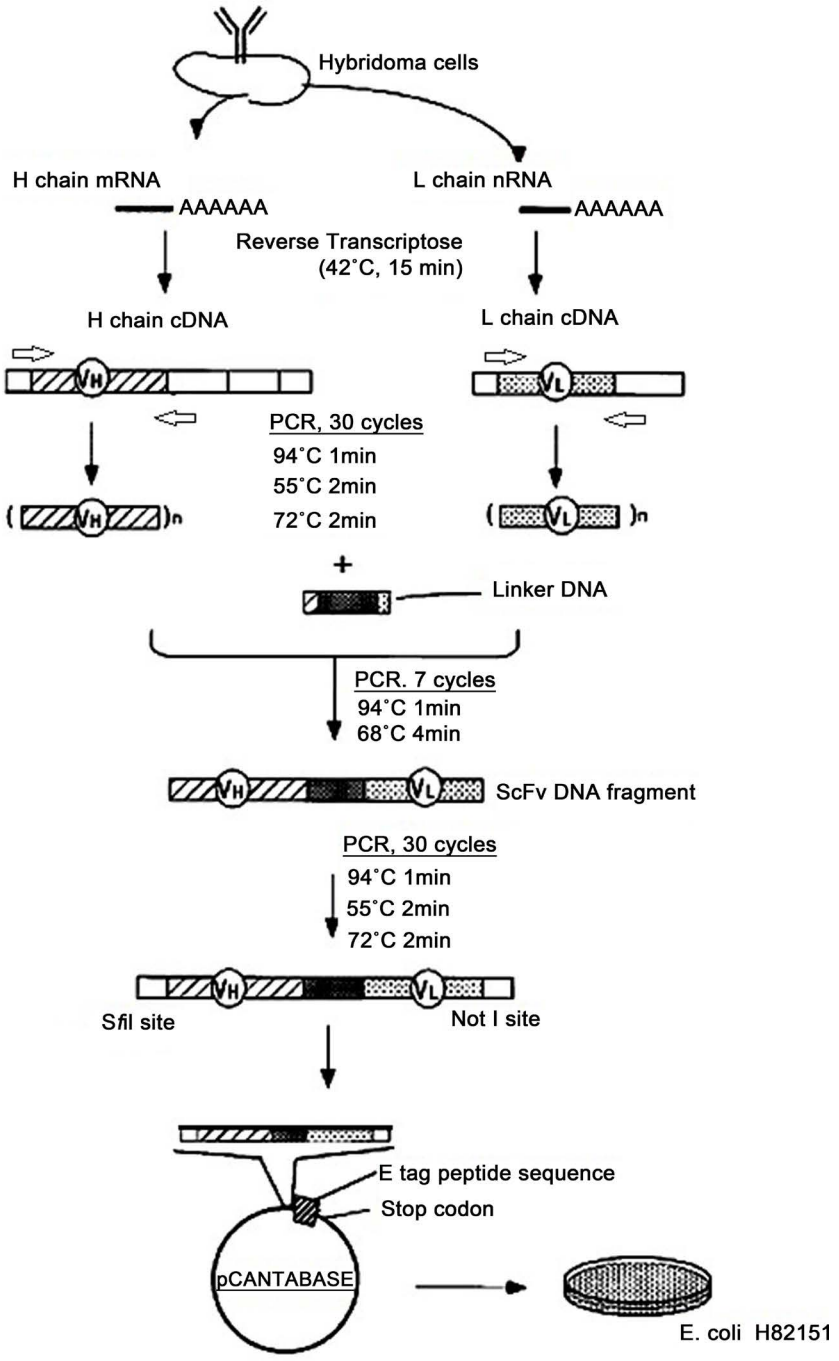

Figure 4. Construction of recombinant scFv antibody using phage display technology [12].

reaction of antibodies collected from the immature pool of antibody is goes per the number of the antibody pool, as such as 10^{6-7} million for a small antibody pool, to 10^{8-10} million for a large collection, constructed by phage display technology [45]. As mentioned earlier, the constructed pool of antibodies has been amplified using V gene sequence as template and VH a VL wide range of primers in RT-PCR are represented specially CDR3s [46]. The regions and degree of wide range of collected antibodies per points of largest species of the antibody

selection. Ultimately, the value of any library is best determined by its success in yielding specific, high-affinity ligands for a variety of target molecules.

Still, antibodies with highest reaction can be constructed to connect with stronger specific antigen sequence by dimer sequences [47]) or in vitro affinity maturation. The wide range antibody DNA sequences may be represented using different methods, such as mutator strains [48], RT-PCR, chain scuffling; gene scuffling, and codon based mutator-genesis. The capability to change the binding reaction strength and specificity by developing monoclonal antibody source is influential development in antibody display technology. Phage-display has been mainly needed to mimic the target protein with antibodies pool, to describe epitopes for parent monoclonal antibodies, to increase antibody reaction, to isolate conjugate and to coat phage-antibody collections [49].

Phage-display technologies have also been needed to give a clear idea of the 3Dimintional protein binding loop that mimicking the antibodies receptors and their insight limited [50]. Antibody-phage display is useful in that it permits the genuine display of scFvs, Fc sequences and diabody-sequences [51].

8. Application of Phage Display Technology

8.1. Epitope Mapping of Antibodies

Phage display system could be utilized in various fields of study. This technology can be used to map specific antibody binding sites and is a simple and relatively low cost approach. Epitope mapping of antibodies, such as anti-HIVgp120 and HBsAg, has been successfully performed using random phage display peptide libraries. Specific epitopes of polyclonal antibodies against Nipah virus was also successfully mapped using a random peptide library of heptamers expressed on bacteriophage (M-13) [52].

Phage display peptide libraries contain millions of foreign sequences, which represent a huge pool of antibody for a diversity of receptor ligands. Peptide selections from random peptide libraries are powerful tools for identifying receptor ligands without knowing its ligand structure. Affinity selections of random peptide libraries against receptors are a potential approach for new drug discovery. Furthermore, selected small cyclic peptides from a phage-antibody pool able to bind with activate target antigen the cytokine protein receptor is advanced. Hence, it can act as a full antagonist and may be a substitute for the larger natural hormone [53].

8.2. Production of Vaccines and Diagnostics

To discover vaccines and diagnostics using phage display technology, antigenic and immunogenic mimicry are two important factors. Peptides selected against an antibody are antigenic mimics of the natural epitopes. If cross-reaction of the new antibodies is elicited against these antigenic mimics with the antigenic determinants of the natural epitopes, such peptides display both antigenic and immunogenic properties [52]. Sali [52] envisaged many uses for this technology such as production of malaria vaccine, which two independent libraries were

panned for wide range of phage-antibody fusion expressed on the surface of bacteriophage with a mAb directed toward malarial antigen, their data showed high affinity of the antibody clones' pool that reacted strongly with the mAb. Moreover, the DNA sequencing of the primers set combined with the templates of mAbs explained the high similarity of the selected clones.

Moreover, phage-display system was used to construct (scFv) antibodies that identification *Clostridium difficile* toxin B, which is a toxic type of *Clostridium* bacteria. Deng [54] produced a highly specific scFv antibody directed toward *C-difficile* toxin B that does not react with samples collected from a toxin B-negative *C-difficile* strain. The affinity of the soluble single-chain antibody is significantly higher than the parent monoclonal antibody as showed in ELISA test, and detect lower reading of 10 ng *C-difficile* B/well. Still, phage display system is a fast and actual advance technology for advance of new resulted immuno-diagnostic antibodies.

9. Expression of Recombinant scFv Protein in Prokaryotic and Eukaryotic Cells

E. coli is excessively used for the construction of antigens fragments, as well as antibody sequences, for example Fab or scFvs. Soluble scFvs are produced by expressing the target antigen DNA sequence toward the *E. coli*-periplasm, where the disulphide amino acids used to express the target antigen in soluble and active shape. The bacterial host manufacture scFv protein normally in small scale of 0.1 - 1 mg/L of yield, when the OD reach 600 of 1, however, the large scale of 10 mg/L can be achieved [55].

However, scFvs active and soluble protein can be expressed in great amount in cytoplasmic space in *E. coli* after eliminating signal sequence from scFv DNA sequence [55]. To collect more folded and active protein from the cytoplasm of the cell, many laboratories had done alteration in the cytoplasmic oxido-redox environment by changing protein of the thiore-doxin and glutare-doxin synthesis [56]. Though, this modification results in production of folded intra-cytoplasm-oxidised protein sequence. Also, the proper folded scFv protein produce in *E. coli* reducing-environment of the cytoplasm is the useful product to be manufacturing that inhibit virus and carry a toxin that lysis cancer cells. Besides, the use of intrabodies leads to many interesting possibilities for gene therapy and the in vivo study of protein function.

Accordingly, Modified *E. coli* cell called Origami (DE3) (Invitrogen) could produce 10 times much better soluble antibody compares with alternative hosts, even though, the production quantity is alike [56]. The Origami *E. coli* have designed thiore-doxin (trxB) beside gluta-thione (gor) gene sequences that activate the creation of disulphide poly peptide in the bacterial cytoplasm. Origami hosts are match with ampicillin-resistant vectors and are perfect with the pET-32 plasmid connected with the thiore-doxin fusion tag that would activate the creation of disulphide linker in the cytoplasm. The pET-32 b(+) vector is designed to produce protein that connected with the a Trx-Tag protein [55]. The multiple

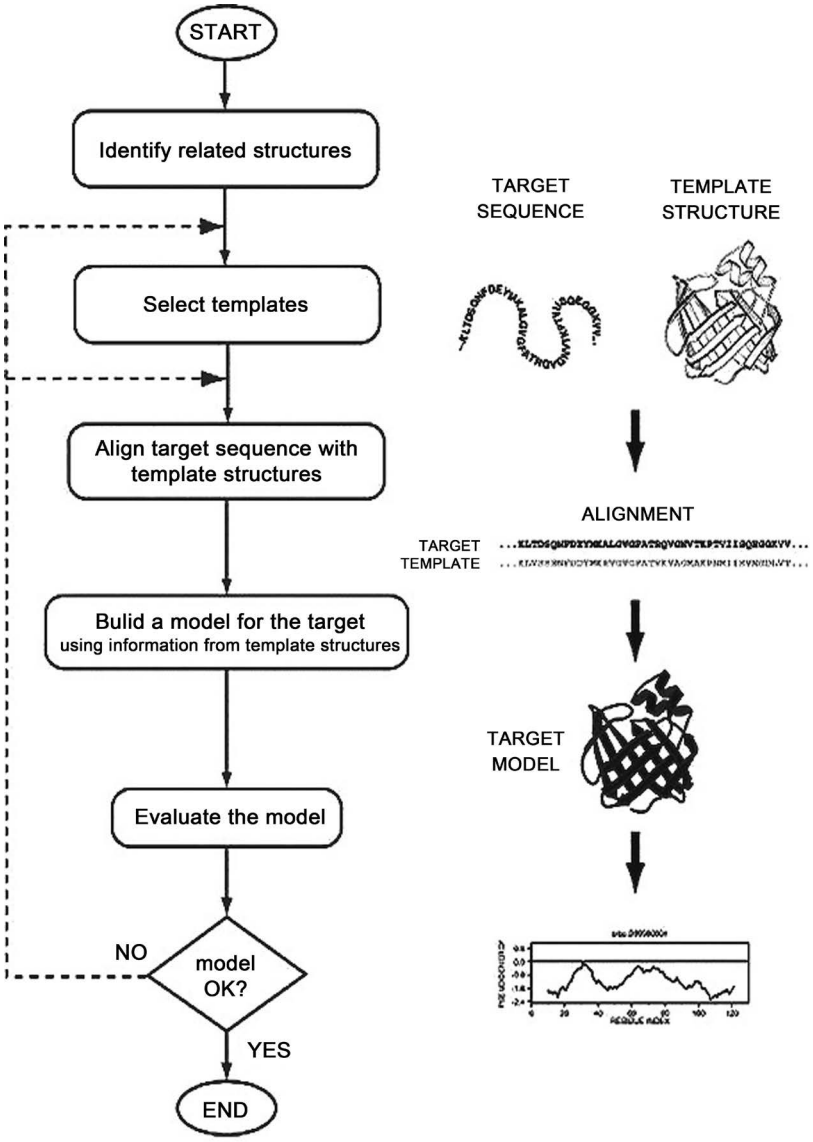

Figure 5. Choosing the related structure and the step following of building correct 3D structure of protein, 3D structure of proteins from the same family is more conserved than their primary sequences [9].

cloning site in the plasmid have His-Tag and S-Tag sequences for characterization and purification. Besides, the sequence referred to as pBR-32 resolution. For example, the T7 production site is inverted on the plasmid sequence. The functional genes areas also mapped on plasmid cycle. Besides, the DNA sequence of the plasmid copied by the T7 RNA-polymerase is described in Figure 5.

10. Protein Preparation Using Affinity-Metal-Chromatography

This system had sort of column which, purify the antibody (plasmid and insert target protein in frame) the plasmid also fused with specific tag that precisely bind to ligand manufactured in the solid part on an unsolvable backing called-matrix. The ligand holds 6x-his-tagged fragments which connects to the

unsolvable backing-matrix (Ni^{2+}) solid that can fuse to His-Bind mastics. After the non-reacted amino acids are drained, the bound amino-acids is captured by elution with imidazole. IMAC is a great system to capture specific target protein. IMAC used to precipitate and purify the scFv protein [55].

For the capture of large amounts of target fragments, many methods have been advanced, as well as production of tagged amino-acids fragments, that capture the target specific protein to be purified by affinity chromatography [50]. However, many fusion proteins, such as glutathione-S-transferase or protein (A) are large proteins; therefore, there is a potential risk of mutation in protein sequence, because of the fusion partner. Additionally, the fusion protein is expressed in a native form for efficient isolation after affinity chromatography [50].

Another successful approach for the purification of fusion proteins is the addition of six consecutive 6xHis-tag to the protein. The specificity of the histidine residues for solid matrix lets the protein-fused tag to hold away from unfused other proteins with high purity using metal chelate affinity chromatography. Because only six residues are fused to the protein, the risks of altering the properties of the protein of interest are minimised. Additionally, purification under denaturing conditions is possible. Several antibodies and chelator/enzyme conjugates are reported to detect histidine-tagged proteins. However, these are restricted to specific expression vectors or recognise histidine-tagged proteins with only reasonable affinity [52].

11. Homology Protein Modelling

The use of homology modelling is to show three-dimensional protein and verify the CDRs as reported by Mahgoub [18]. Phage display can be used with molecular modelling and site-specific randomisation to advance the ways of capture the target antigens using Mabs. Prediction of three-dimension structure of target unknown protein is described. The structure would build per the highest sequence similarity to a correlated published known protein structure, which is called the template [56]. There are a few steps needed to be fallow to build a reliable model: 1) the chosen structure shall be when the proteins sequence alignment (target and templates) is correct; 2) template-target chosen with higher similarity. The structure prediction would be effected with any change in proteins (template-sequence alignment) sequences is possible since any difference in these sequences would result in totally different 3D structure as shown in **Figure 5** also described by Lesk and Chothia [9]. It is also simplified the search for the best candidate 3D structure's as the chosen model from related family sequences would be well-maintained than their own sequences (Lesk and Chothia [9]. Accordingly, the sequences alignment would show alike also the predicted 3D models will be similar. However, in some rare cases some poor aligned sequences would have a similar 3D structure. Mahgoub [15] predicted models become the reliable 3D models comparable structure with precise insight in compare to model prediction decided of wet laboratory experiment characterization

of the protein affinity and specificity.

Single Chain Fragment Variables Antibody could be manufacture in divalent and trivalent complexes which are easier in folding and 4 times soluble than the mono-valet's complexes. Also, large scale production of the antibody in yeast cells and mammalian cells hosts can be recommenced to further characterize the scFv antibody. Beside in bioinformatics direction, the EGFR model can be docked against the antibody molecule to visualize the binding sides and to produce minimized energy antibody structure that can used for stronger antigen antibody binding.

In conclusion: Single chain fragment variable is great addition to modern drug industry, which is tags to drugs and works as a diagnosis agent and a vaccine that disabled breast cancer antigens.

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List of Abbreviation

AP	alkaline phosphatase
CL	constant light domain
CH1	constant heavy domain 1
CH2	constant heavy domain 2
CH3	constant heavy domain 3
CH4	constant heavy domain 4
CDRs	complementary determining regions
CEA c	himeric anti–carcino-embryonic antigen
cDNA	Complementary determine regions
CDC	Complement dependent cytotoxicity
C3A8	mouse B-cell hybridoma cell line
CMV	cucumber Mosaic Virus
dNTP	Deoxyribonucleotide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERRAT	novel structure evaluation programme
Fab	Fragment antigen binding
Fc F	ragment crystalline
GlY	Glycine
6x his-tag	6x histidine-tagged
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL2	Interleukin2
IMAC	Immobilized Metal Affinity Chromatography
gB	Glycol proteinB
gH	Glycol protein
HIV	Human immunodeficiency virus
HBsAg	Hepatitis B virus surface antigen
Kg	Kilo gram
MCF-7	Michigan cancer foundation-7
M13KO7	helper phage
mM	Milimolar
µl	microlitre
µg	microgram
Mab	Monoclonal antibody
MR1	affinity-matured derivative-1
mRNA	messenger ribonucleic acid
PCR	Polymerase chain reaction
RT-PCR	Reverse Transcriptase-Polymerase chain reaction

protein 3D	Protein three dimensional
RE	Restriction endonuclease
scFv	Single Chain Fragment Variable
Ser	serene
trxB	thioredoxin reductase part B
OD	Optical Density
VL	Hyper variable light chain
VH	Hyper variable heavy chain

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