

Proteomic response of marine-derived *Staphylococcus cohnii* NIOSBK35 to varying Cr (VI) concentrations

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Abstract

Chromium in its hexavalent state is a water-soluble and toxic element to living organisms present in the environment. However, some organisms are resistant and reduce the toxic forms of Cr (VI) to less toxic or non-toxic forms. A global proteomic analysis of *Staphylococcus* sp. NIOSBK35 under different chromate concentrations (0, 100, 200 and 300 mg L⁻¹) at different time points in its growth stages (6, 9, 12, 18, 24 and 36 h) resulted in the identification of 878 proteins. Of all the proteins expressed, 13 proteins [23 rDNA (uracil-5-) methyltransferase RumA, multidrug ABC transporter ATP binding protein, dihydroxy acid dehydratase, polysaccharide biosynthesis protein, etc.] were expressed only in the presence of chromium. 14 proteins were up-regulated in response to chromium (VI), namely, alkyl hydroperoxide reductase, ATP- dependent Zn metalloproteinase, hsp90- like protein, NAD (P)- dependent oxidoreductase, etc. Most of the proteins involved in normal cell functioning like 1-pyrroline-5-carboxylate dehydrogenase, ribosomal proteins (30S ribosomal protein S11, 30S ribosomal protein S2, 50S ribosomal protein L32), aconitate hydratase, DNA primase, serine- tRNA ligase, phosphoenolpyruvate-protein phosphotransferase, enolase, sulfur transferase FdhD, etc. were found to be down-regulated. On grouping these proteins into their COG (Cluster of Orthologous Groups) functional categories, these were found to be involved in translation, carbohydrate metabolism, stress proteins, amino acid transport and membrane transport mechanisms. The proteomic response given by *Staphylococcus* sp. NIOSBK35 did not show expression of Cr-specific proteins, indicating towards a different mechanism of Cr-tolerance as the organism was able to survive and grow at high concentrations of Cr (VI).

Keywords: Cr (VI), protein expression, peptide mass fingerprinting, LC-MS-QTOF

Introduction

Chromium is the element located in group VI-B (transition elements) of the periodic table and can exist in various oxidation states (-2 to +6). However, Cr (VI) and Cr (III) are the most stable forms in the environment.¹ Hexavalent chromium is a strong oxidising agent and is reduced to trivalent chromium in the presence of organic matter. However, in the presence of oxygen, Cr (III) is converted to toxic Cr (VI).² Chromium can occur naturally as lead chromate and potassium dichromate.³ Cr (VI) can enter the environment from the anthropogenic sources such as textile dyeing, chemical and pigment production, wood preservation, electroplating, tanning, coating alloys like stainless steel.⁴

Cr (VI) is highly soluble in aqueous solvents such as water which enables it to cross cell membranes and cause damage to the cellular constituents.⁵ Chromium is known to have carcinogenic and mutagenic effects on biological organisms.⁶⁻⁸ It reacts with intracellular reductants like glutathione to generate free radicals and reactive oxygen species (ROS).⁹ Due to its hazardous nature, Environmental Protection agencies have established permissible limits for Cr (VI) discharge into the water bodies¹⁰ and hence, efficient methods for treating chromium-containing wastes is important.¹¹

Various methods have been employed for chromium removal from the environment, including chemical oxidation or reduction, electrochemical treatments, precipitation.¹² An alternate approach was the use of microorganisms to reduce chromium toxicity.¹³ Microorganisms possess various mechanisms for resistance and detoxification of Cr (VI) like periplasmic bio-sorption, intracellular bioaccumulation, biotransformation, efflux mechanism.^{8,14,15} Reports on biotransformation of Cr (VI) in bacteria include *Stenotrophomonas maltophilia*,¹⁶ *Cellulosimicrobium* sp.,¹⁷ *Alishewanella* sp. WH16-1.¹⁸

Huge amount of data is available regarding chromate tolerance, reduction by chromate reductase^{13,17} and the genes involved in chromium tolerance.¹⁹ However, the exact mechanism or the metabolic pathways involved in bacterial survival at high concentrations of Cr (VI) and also the harmful effects of chromate on normal cell functioning are unknown. This study focuses on identifying different proteins expressed (proteomic response) for chromium tolerance and elucidating the metabolic pathways affected under varying Cr (VI) stress in marine-derived *Staphylococcus* NIOSBK35 by peptide mass fingerprinting (LC/ MS- QTOF).

Materials and Methods

Growth characteristics of chromium (VI) tolerant #NIOBK35

Staphylococcus cohnii #NIOBK35 isolated from sediments collected from mangroves at Karwar (14.49°N, 74.09°E) tolerant to 1000 mg L⁻¹ arsenite,²⁰ was also tolerant to 500 mg L⁻¹ Cr (VI) supplemented in Luria Bertini (LB) broth. The synchronous culture was inoculated in experimental flasks containing LB broth (HiMedia, India) in seawater supplemented with varying Cr (VI) concentrations, viz., 100, 200 and 300 mg L⁻¹, respectively. An experimental culture control without Cr (VI) was maintained. Another set of three control flasks of LB with Cr (VI) without the culture were also kept as blank [this was done, since, the addition of 100, 200 and 300 mg L⁻¹ Cr (VI) resulted in change in colour of the broth to orange with varying intensities (since potassium dichromate was used as a source of Cr (VI)) depending on the concentration. Also, the total volume of all the flasks was kept constant]. All the experimental flasks were incubated at 28 °C at 120 rpm for about 48 h (approx. till culture reached its stationary phase). Absorbance at 600 nm was measured using UV Visible Spectrophotometer (Genesys 20, Thermo Scientific, USA) every 3 h, followed by harvesting of cell pellets for whole cell protein extraction.

For whole cell protein extraction, quantification of proteins, in solution digestion of proteins and peptide mass fingerprinting, the methodology was followed as given by Shah and Damare²⁰.

Proteomic response of #NIOBK35 to Cr (VI) stress

Identified proteins were further analysed for their regulation using Mass Profiler Professional Ver. 14.9.1 (GeneSpring GX, Agilent Technologies, USA). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012993. The samples were grouped based on the Cr (VI) concentration that they were subjected to (100, 200 and 300 mg L⁻¹) and then regrouped based on the time points at which the cell pellets were harvested during the growth of the culture. A fold change cut-off of 2.0 [fold change cut-off was applied on the absolute fold change value of the log₂ normalised data, and the log FC (log fold change) value was finally used to represent the expression levels of the proteins] and a *p*-value of 0.2 was maintained for analysing the regulation of proteins. For comparing up- and down-regulation of proteins expressed at different concentrations, a Venn diagram was constructed as 100 vs 0 mg L⁻¹, 200 vs 0 mg L⁻¹ and 300 vs 0 mg L⁻¹ Cr (VI). Similarly, Venn diagrams were constructed for time point comparison at different Cr (VI) concentrations, i.e., 100 vs 0 mg L⁻¹, 200 vs 0 mg L⁻¹ and 300 vs 0 mg L⁻¹ at 6, 9, 12, 18, 24 and 36 h, respectively. Venn diagrams revealed common as well as unique proteins to a particular condition.

The up- and down-regulated proteins were further categorised into their respective metabolic pathways using KEGG (Kyoto Encyclopedia for Genes and Genomes) pathway ver. 37 database.²⁰

Results

Peptide mass fingerprinting

A total of 878 proteins were identified across all Cr (VI) concentrations (0, 100, 200, 300 mg L⁻¹) and, all time points (6, 9, 12, 18, 24 and 36 h).

Proteomic response of #NIOBK35 to Cr (VI) stress

Effect of Cr (VI) concentration on protein regulation

All 878 proteins were considered for determining their regulation. Fold change was calculated for each pair of conditions [100 vs 0 mg L⁻¹, 200 vs 0 mg L⁻¹ and 300 vs 0 mg L⁻¹ Cr (VI)]. This resulted in filtering out of 31 proteins. Thirteen proteins were present only in Cr (VI) containing samples (100, 200 and 300 mg L⁻¹) and absent in control (Table 1). On comparison of proteins expressed at 100, 200 and 300 mg L⁻¹ Cr (VI) against control (0 mg L⁻¹), a total of 847 proteins were found to be up- and down-regulated. Of the total proteins, 113, 318 and 193 proteins were up-regulated at 100, 200 and 300 mg L⁻¹ Cr (VI) respectively against control [without Cr (VI)]. Correspondingly, 457, 236 and 306 proteins were found to be down-regulated at 100, 200 and 300 mg L⁻¹ Cr (VI) respectively against in the absence of Cr (VI). Of the total up-regulated proteins across all the three different concentrations, 14 proteins were found to be up-regulated at all the Cr (VI) concentrations (Table 2 and Supplementary data 1). 51, 186 and 89 proteins were found to be exclusively up-regulated at 100, 200 and 300 mg L⁻¹ Cr (VI) (Figure 1a). Of the total down-regulated proteins across the three different concentrations, 194 proteins were down-regulated across all the concentrations (Table 2 and Supplementary data 2) while, 161, 5 and 29 proteins were found to be uniquely down-regulated to 100, 200 and 300 mg L⁻¹ Cr (VI) respectively (Figure 1b). There were some up- and down-regulated proteins that were expressed in two conditions, as shown in Figure 1a and 1b.

Effect of Cr (VI) concentration on growth

Time point comparison across the concentrations was made which too revealed a unique set of proteins at particular concentrations and also the proteins common across the three concentrations when compared against the absence of Cr (VI) at that particular time point (Supplementary data 1, 2 and 3). At 6, 9, 12, 18, 24 and 36 h, it was found that 13, 9, 6, 9, 2 and 5 proteins respectively were commonly up-regulated, i.e., across all three Cr (VI) concentrations as in the absence of Cr (VI)

(Supplementary data 1 and 3). Likewise, 35, 157, 176, 90, 154 and 26 proteins were down-regulated across all three Cr (VI) concentrations at 6, 9, 12, 18, 24 and 36 h respectively (Supplementary data 2 and 3).

Expressed proteins classified into their respective pathways

The filtered 847 proteins showing variable expression were grouped into their respective metabolic pathways depending on their functions (COG functional categories) using the KEGG pathway. Maximum proteins were belonging to hypothetical proteins (137), followed translation (100), carbohydrate metabolism (90), stress proteins (88), amino acid metabolism (78), membrane transport (59), metabolism of cofactors and vitamins (44), and so on (Figure 2).

Discussion

Cr (VI) is structurally similar to sulphates (isostructural anions),²¹ probably they get actively transported through the cell membranes via sulphate transporters.²² Cr-resistance mechanisms such as plasmid-mediated resistance,^{1,23} biosorption,²⁴ and efflux pump²⁵ have been reported in many Cr (VI) tolerant bacteria. Other resistance mechanisms involve the synthesis of ROS scavenging enzymes such as catalase, superoxide dismutase, SOS response for DNA repair by RecA, RuvAB and RecG proteins, etc.²⁶ It has been reported that in *Pseudomonas* spp., the efflux pump mechanism is mediated by chromate transporters (Chr).²⁵

The current study focused on the expression of proteins by *Staphylococcus* sp. NIOSBK35 in response to varying Cr (VI) concentrations. It was observed that more proteins were up-regulated at 200 mg L⁻¹ Cr (VI) as compared to 100 mg L⁻¹ Cr (VI) and 300 mg L⁻¹ Cr (VI). Similar observations about the growth enhancement in the presence of chromate have been reported previously by Henne et al.²⁷ Most of the metal-related proteins were up-regulated indicating the stress exerted by metal on the metabolism of the organism. Proteins like 23 rDNA (uracil-5-) methyltransferase RumA involved in translation mechanism were found only in the presence of Cr (VI). Similarly, membrane transport proteins such as ABC transporter (plasmid), multidrug ABC transporter expressed in the presence of Cr (VI) could be possibly involved in the transportation of Cr (VI) across the cell membrane. Interestingly, normal cellular proteins involved in lipid metabolism (CDP-glycerol- poly (glycerophosphate) glycerophosphatase, esterase), carbohydrate metabolism (beta-galactosidase, phosphoglyceromutase, phosphotransacetylase), amino acid metabolism (dihydroxy acid dehydratase, prephanate dehydrogenase), cellular community- prokaryotes (glutamyl endopeptidase precursor, serine proteinase SspA, polysaccharide biosynthesis protein) were detected only in the presence of Cr (VI) indicating enhanced cellular activity in response to cope up with Cr (VI) stress.

Chromium-induced proteomic response in *Staphylococcus* has shown up-regulation of alkyl hydroperoxide reductase, 30S ribosomal protein S1 ABC transporter²⁸, which was also observed in our study. Cerventes et al.¹ have reported that the function of Chr protein which is involved in extruding Cr (efflux mechanism) out of the cell is similar to that of ArsB (membrane protein involved in arsenic resistance which extrudes arsenite). *Staphylococcus* sp. NIOSBK35 showed up-regulation of arsenical pump-driving ATPase and arsenical pump membrane protein.²⁰ Proteins like FMN reductase (NADPH),²⁹ NAD (P)-/ NAD (P) H-dependent oxidoreductase,³⁰ nitroreductase³¹ responsible for chromium reduction were also found to be up-regulated. Hence, these proteins could be responsible for repulsing chromium from the cell.

Proteins like heme ABC transporter ATP-binding protein, heme-binding protein, ferritin involved in iron transport and iron binding, sulfite reductase (sulfur metabolism) reported to be a part of bacterial mechanism in chromate resistance,³² superoxide dismutase,^{29,33} alkaline shock protein, lipid hydroperoxide peroxidase, molecular chaperone GrpE, peroxiredoxin, universal stress protein UspA were down-regulated, indicating towards a dissimilar mechanism for Cr (VI) tolerance by *Staphylococcus* sp. NIOSBK35. Most of the DNA replication and repair proteins have been reported to be up-regulated to combat the chromium stress in different organisms.^{27,32} However, contrasting results were obtained in the current study. Proteomic analysis in *P. aeruginosa* and *P. putida* F1 did not reveal any significant regulation change in proteins involved in DNA replication and repair mechanisms against Cr (VI) stress³⁴⁻³⁶, which was also observed in our study.

Henne et al.²⁷ have reported the altered expression of a large number of proteins in *Arthrobacter* sp. strain FB24, most of which were involved in amino acid transport and metabolism, carbohydrate transport and metabolism, and energy production and conversion. Similar results were obtained in the current study, which indicates the deleterious effect on normal cell functioning. Proteins like ATP synthase subunits, fructose 1, 6- biphosphatase were down-regulated, which is in contrast to the results obtained in *Pseudomonas aeruginosa* where these proteins were overproduced in the presence of Cr (VI).³⁴ Most of the proteins expressed belong to the group of hypothetical proteins (proteins whose functions are hypothesised not proven). This could be because the database is not well curated. Most of the proteins involved in translation were affected under Cr (VI) stress, followed by proteins involved in carbohydrate metabolism, amino acid metabolism, membrane transport, metabolism of cofactors and vitamins, nucleotide metabolism, replication and repair, etc.

Bacteria can tolerate Cr (VI) because of the presence of chromate efflux mechanism.¹¹ However, no such proteins were detected in our study, indicating towards a likely alternate mechanism for tolerance of Cr (VI) in the absence of well-described chromate specific mechanisms. Certain

enzymes such as flavoenzymes are known to be responsible for the reduction of arsenate, chromate, sulphate, etc.¹⁸ Nevertheless, such mechanisms and the proteins responsible need to be studied further since there is no report on other bacterial mechanisms for Cr (VI) tolerance. The genome-wide analysis will enable us to study the specific proteins responsible for Cr (VI) tolerance. One of the anomalies reported in this study was up-regulation of many proteins at 200 mg L⁻¹, and down-regulation of the same at 100 and 300 mg L⁻¹, which could be addressed using quantitative proteomic approach, as the data in the present study is expressed as relative values and not as absolute values. The identification of specific proteins could be improved further by using the annotated whole genome of this bacterium as a database for identification of the proteins.

The Cr (VI) bacterium discussed in this study could be a potential isolate for bioremediation of chromium-contaminated effluents like generated from tanneries and chrome-plating industries, due to its ability to tolerate up to 500 mg L⁻¹ Cr (VI). Similar Cr-tolerant bacteria have been tested for remediation of tannery effluent, where reduction in the levels of Cr (VI), COD, BOD, TDS, TSS, phenolics have been reported.³⁷⁻⁴⁰ *Staphylococcus cohnii* NIOSBK35, reported in this study has also been checked for bioremediation of tannery effluent, where it was able to reduce the COD by 45% and toxicity by 50% (unpublished work).

Conclusion

A significant amount of data is available on the tolerance and reduction mechanisms exhibited by different organisms against chromium stress, in terms of chromium-specific genes and proteins expressed. However, in the current study, no chromium-specific proteins were detected, yet the organism was found to not only tolerate, but grow and express a significant number of proteins, with variable levels. Most of the proteins involved in normal cell functioning were found to be down-regulated, which shows the toxic effects of Cr (VI) on the organism. The differential expression of the proteins indicates a possible non-chromium specific mechanism for Cr (VI)-tolerance in *Staphylococcus* sp.

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Conflict of Interest

Both authors state that there is no conflict of interest.

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Legend to figures

Figure 1. (a) Venn diagram showing total number of up-regulated proteins at 100, 200 and 300 mg L⁻¹ when compared against control without Cr (VI) (b) Venn diagram showing total number of down-regulated proteins at 100, 200 and 300 mg L⁻¹ when compared against control without Cr (VI). [A- List of proteins from 100 mg L⁻¹ against control without Cr (VI), B- List of proteins from 200 mg L⁻¹ against control without Cr (VI), C- List of proteins from 300 mg L⁻¹ against control without Cr (VI)].

Figure 2. Bar chart illustrating the metabolic classification of the expressed proteins

Table 1: List of proteins expressed only in the presence of metal and the metabolic pathways they are involved

Protein name	Function (KEGG)	Metabolic pathway (KEGG)	Accession number (NCBI)
23 rDNA (uracil-5-) methyltransferase RumA	Ribosomal protein	Translation	1032138401
ABC transporter (plasmid)	ABC transporters	Membrane transport	563257329
beta- galactosidase	Galactose metabolism	Carbohydrate metabolism	1113040337
CDP-glycerol- poly (glycerophosphate) glycerophosphatase	Glycerophospholipid metabolism	Lipid metabolism	1113041787
Dihydroxy acid dehydratase	Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	1032139789
Esterase	Glycerolipid metabolism	Lipid metabolism	1032139184
Glutamyl endopeptidase precursor, serine proteinase SspA	Quorum sensing	Cellular community-prokaryotes	815785678
Multidrug ABC transporter ATP binding protein	ABC transporters	Membrane transport	1032204569
Phosphoglyceromutase	Glycolysis	Carbohydrate metabolism	1072350828
Phosphotransacetylase	Pyruvate metabolism	Carbohydrate metabolism	1032204158
Polysaccharide biosynthesis protein	Polysaccharide biosynthesis	Cellular community-prokaryotes	1101915694
Prephanate dehydrogenase	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism	1101909743
Uncharacterized protein	Uncharacterized protein	Uncharacterized proteins	1072350321

Table 2: List of proteins classified based on their metabolic pathways (KEGG database) whose regulation pattern was significantly changed in the presence of Cr (VI) at 100, 200 and 300 mg L⁻¹, relative to control

Protein name	Fold change of proteins at a Cr (VI) concentration against absence of Cr (mg L ⁻¹)			Function (KEGG)	Accession number (NCBI)
	100	200	300		
Amino acid metabolism					
1-pyrroline-5-carboxylate dehydrogenase	-2.803	-1.157	-2.284	Alanine, aspartate and glutamate metabolism	1143274836
Type I glutamate- ammonia ligase	-2.620	-0.734	-1.266	Arginine biosynthesis	1032135231
Serine hydroxymethyl transferase	-2.847	-0.224	-0.698	Glycine, serine and threonine metabolism	1032202817
Carbohydrate metabolism					
Phosphoglucosamine mutase	0.364	1.127	0.402	Amino sugar and nucleotide sugar metabolism	1032202767
UDP- N- acetylglucosamine 1- carboxyvinyltransferase	1.526	2.369	0.856	Amino sugar and nucleotide sugar metabolism	1032202829
Acetate- CoA ligase	0.256	1.497	2.332	Pyruvate metabolism	1032136219
Aconitate hydratase	-1.679	-0.425	-0.744	Citrate cycle	1032205520
Dihydrolipoamide succinyl transferase	-0.126	-1.549	-1.265	Citrate cycle	1032139956
Enolase	-3.217	-0.160	-1.055	Glycolysis	1032203973
Phosphogluconate dehydrogenase (NADP(+)-dependent, decarboxylating)	-1.975	-1.567	-2.218	Pentose phosphate pathway	1032205373
Acetate kinase	-1.734	-0.813	-1.873	Pyruvate metabolism	1032205194
Phosphoenolpyruvate-protein phosphotransferase	-0.270	-1.064	-0.235	Pyruvate metabolism	807191994
Cell wall biogenesis					
Teichoic acid biosynthesis protein	1.958	1.044	0.418	Teichoic acid biosynthesis	807190859
Nitric oxide dioxygenase	-2.796	-1.811	-3.427	Nitrogen metabolism	1113039696
Sulfur transferase FdhD	-1.264	-1.197	-3.301	Sulfur metabolism	1032138506
Lipid metabolism					
beta- ketoacyl- [acyl-carrier-protein] synthase II	0.919	1.789	1.311	Fatty acid biosynthesis	1032137339

Enoyl- ACP reductase	0.595	1.909	2.812	Fatty acid biosynthesis	1032203836
Metabolism of cofactors and vitamins					
Peroxiredoxin	-2.42	-0.71	-3.24	Glutathione metabolism	1032139694
Nucleotide metabolism					
Uracil phosphoribosyl transferase	-1.145	-0.066	-0.961	Pyrimidine metabolism	1032136188
Replication and repair					
DNA primase	-0.746	-0.067	-5.165	DNA replication	1032203286
Stress proteins					
Peptidase M28	0.212	3.125	2.869	Hydrolases	1032137745
NAD (P)-dependent oxidoreductase	3.781	4.173	0.898	Oxidoreductases	748778870
Alkyl hydroperoxide reductase	1.282	0.305	2.777	Stress protein	1101915785
ATP- dependent Zn metallopeptidase	1.999	0.227	0.882	Stress protein	1072350045
Hsp90- like protein	1.454	0.855	2.338	Stress protein	1072352254
Translation					
30S ribosomal protein S1	0.949	1.199	0.735	Ribosomal proteins	1101914767
30S ribosomal protein S18	0.360	2.196	0.381	Ribosomal proteins	1101916778
50S ribosomal protein L18	0.023	1.814	0.592	Ribosomal proteins	1032204826
Serine- tRNA ligase	-2.847	-3.647	-2.756	Aminoacyl tRNA biosynthesis	1102013349
30S ribosomal protein S15	-3.310	-1.320	-1.539	Ribosomal proteins	1032135288
30S ribosomal protein S2	-2.674	-0.325	-1.301	Ribosomal proteins	1032139250
50S ribosomal protein L16	-1.377	-1.515	-2.262	Ribosomal proteins	1072352343
50S ribosomal protein L32	-2.889	-1.439	-2.866	Ribosomal proteins	1032139304

(Negative values indicate down-regulation of the expressed proteins)

Figure 1:

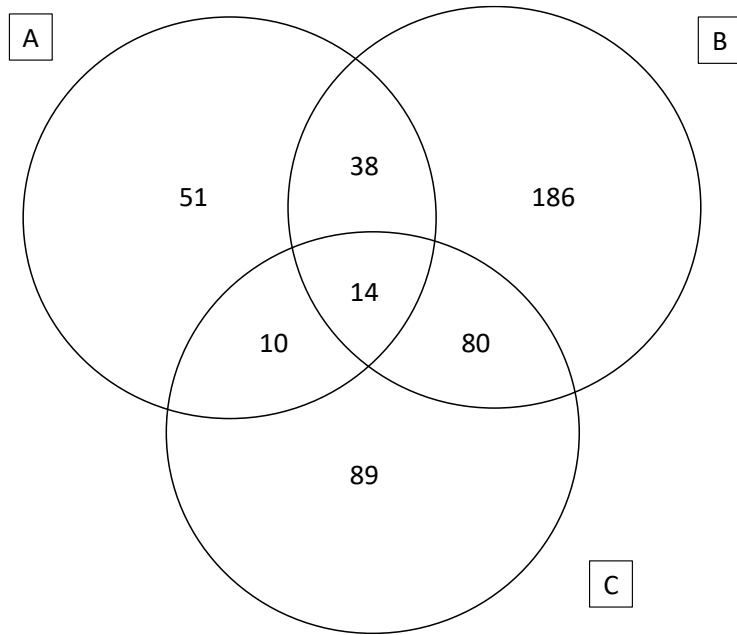


Figure2:

