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Relation between Chlamydia - Like Microorganism 'SimkaniaNegevensis' and Patients Undergoing Hemodialysis

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Abstract

Background: Chronic kidney disease (CKD) has been recognized worldwide public health problem, and the associated morbidity and mortality in patients reaching end-stage renal disease (ESRD) is constantly increasing. Despite the significant improvements and advanced dialysis technologyInfection is a common complication of hemodialytic treatment. Many of these infections are due to sepsis, primarily arising from the vascular access site. *Simkanianegevensis* (S. negevensis) is an obligate intracellular bacterium belonging to the family Simkaniaceae in the Chlamydial order, able to survive and grow as an amoeba resisting microorganism in trophozoites and cysts of Acanthamoeba and other free-living protozoa, which probably represent its natural reservoirs *S. Negevensis*was first defined in 1993. The aimwas to assess diagnosis ofSimkaniaNegevensis infection in hemodialysis patients. **Methods:**This cross-sectional study was performed on 78 hemodialysis patients, and they were treated with regular hemodialysis.Patients were subjected to the following: complete history taking, full clinical assessment, laboratory investigation, (complete blood count, Measurement of serum IgG antibody against *S.negevensis*in hemodialysis patients and Water samples were collected from hemodialysis circuits for detection the occurrence of *S. Negevensis*infection. **Results:** Total of 8 water samples were collected from HD tap water in different occasions after the specific treatment of disinfection from March to November 2020. In the first 4 months of our study when the water samples were negative for *Simkania*, 39 patients were selected randomly, IgG antibodies were measured in their serum and the routine laboratory investigations were established for these patients. When the negative water samples turned positive, we measured the serum IgG for all the patients included in our study (78 patients), and the routine laboratory investigations were also established for all patients. Regarding age and sex distribution among studied group. Mean Age was 50 years distributed as (46.71±10.61 SD) with minimum 23 years and maximum 67 years, regarding sex distribution, most cases were males with 88.5% and females were 11.5 There was no difference in the significance of age and gender between the patients undergoing hemodialysis with negative water samples for Simkania compared to hemodialysis with positive water for S.Negevensis. Regarding HTN and DM distribution among studied groups, there was no significant difference between the patients undergoing hemodialysis with negative water samples for *Simkania* compared to hemodialysis with positive water for *S.Negevensis.* There was no significance in our study regarding hemoglobin between patients who were undergoing hemodialysis with positive and negative water samples for Simkania, Conclusion**:** Our study detected the occurrence of *S.negevensis* in hemodialysis patients. When the patients were undergoing hemodialysis with positive water for *Simkania*.

Key words: Serological Study- SimkaniaNegevensis- Hemodialysis.

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Introduction:
Chronic kidney disease (CKD) has been recognized worldwide public health problem, and the associated morbidity and mortality in patients reaching end-stage renal disease (ESRD) is constantly increasing. Despite the significant improvements and advanced dialysis technology[(1)]
Infection is a common complication of hemodialytic treatment. It has been identified as the second cause of death among hemodialysis (HD) patients and hospitalization for infection in the HD population has increased in the last decades [(2)]
HD patients are the most susceptible to Infection and its complication. They found *SimkaniaNegevensis* for the first time in hemodialysis, and they expected that the water used in hemodialysis could be a cause of infection with this bacterium without clinical risk included for the patients[(3)]
The seropositivity to *S. negevensis* in healthy population groups suggest that the organism is a simple colonizer [(4)]
Angeletti et al. [(5)]examined the occurrence of *S. negevensis* in two HD population, characterized by high susceptibility to infectious complications. They detected for the first time the occurrence of *S. negevensis* in hemodialysis and suggested that water used in hemodialysis could be one of the possible sources of *S. negevensis* infection, without clinical involvement risk for patients.
The study aimed to assess diagnosis of SimkaniaNegevensis infection in hemodialysis patients.

Patients and Methods

This cross sectional study was performed at the hemodialysis unit of Al Mabara health insurance hospital in Zagazig city from March to November 2020.

Seventy-eight hemodialysis patients were enrolled into the study, and they were treated with regular hemodialysis for at least three months. Total urea clearance (Kt/V) was measured to assess the adequacy of dialysis, and it was more than 1.2 in all hemodialysis patients. Exclusion criteria included patients with malignant tumors, patients with HIV infection and patients on immunosuppressant drugs.

Methods:
Patients were subjected to the following:

a) **Complete history taking**
- Age of the patient.
- Sex.
- Respiratory diseases, gastroenteric disorders and history of antibiotic regimen prophylactic and therapeutic.

c) Laboratory investigations:

1) Complete Blood Count

- **<u>Specific investigation</u>**:
  - Measurement of the patient's serum level ofIgG antibody against *SimkaniaNegevensis.*
    - **Assay Principle**: This kit is used to assay the Immunoglobulin G (IgG) in the sample of human's serum, blood plasma, and other related tissue Liquid.
    - **Test principle**:
      1. The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Immunoglobulin G (IgG)in samples.

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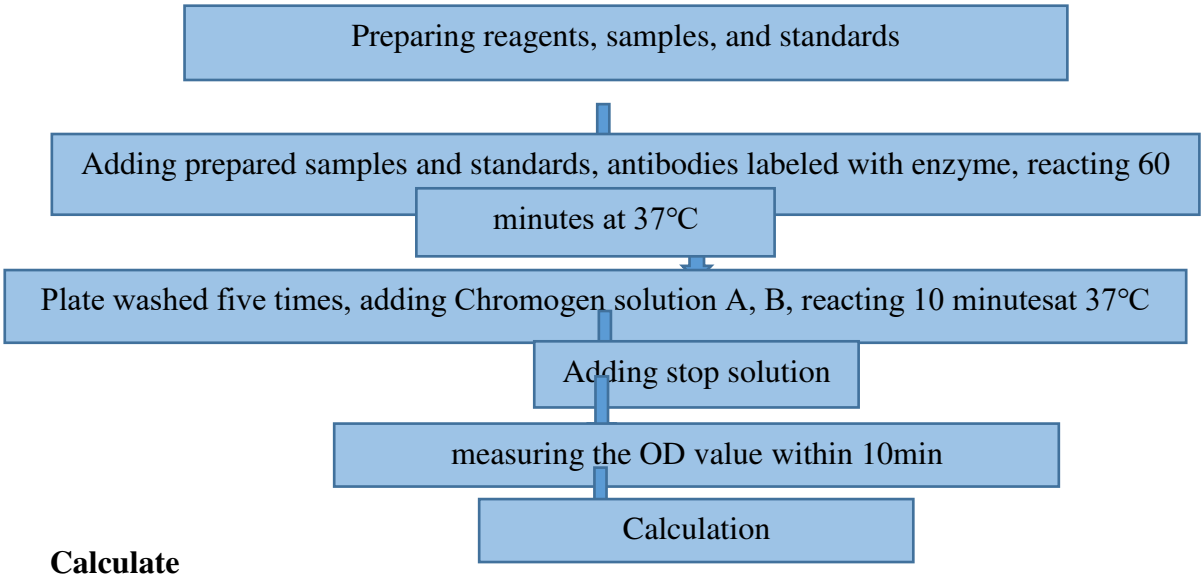
2. We added Immunoglobulin G (IgG)to monoclonal antibody Enzyme well which is pre-coated with Human Immunoglobulin G(IgG) monoclonal antibody, incubation.
3. We added Immunoglobulin G (IgG) antibodies labeled with biotin and combined with Streptavidin-HRP to form immune complex; then carried out incubation and washed again to remove the uncombined enzyme.
4. Then we added Chromogen Solution A, B, the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of color and the concentration of the Human Substance Immunoglobulin G (IgG)of sample were positively correlated.

Summary procedure



Calculate

Taking the standard density as the horizontal， the OD value for the vertical, drawing the standard curve on graph paper, finding out the corresponding density according to the sample OD value by the Sample curve (the result is the sample density)

SensitivityAssay range

Sensitivity: **0.05mg/ml** (The sensitivity of this assay, was defined as the lowest protein concentration that could be differentiated from zero. It was determined by sub tracing two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.) Assay range: **0.05mg/ml→15mg/ml**

Specificity

This assay has high sensitivity and excellent specificity for detection of IgG. No significant cross-reactivity or interference between IgG and analogues was observed.

※ Sample linear regression with the expected concentration of the correlation coefficient R is over 0.95 Intra-assay Precision: 3 samples with low, middle, and high-level human IgG were tested 20 times on one plate, respectively. Inter-assay Precision: 3 samples with low, middle, and high-level human IgG were tested on 3 different plates, 8 replicates in each plate.

CV (%) =SD/meanX100

Intraassay<10%

Inter-Assay: CV<12%

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❖ **Water samples analysis**

In our study, Total of 8 water samples were collected from HD tap water in different occasions after the specific treatment of disinfection using the easy kit during the study time from March to November.

Easy kit contents

- *S.Negevensis* specific primer/probe mix (BROWN) Onceresuspended the kits should remain at -20ºC until ready to use.
- Lyophilized oasis Master mix
- Lyophilized oasis Master mix resuspension buffer (BLUE lid)
- *S.Negevensis* positive control template (RED lid)
- Internal extraction control DNA (BLUE lid)
- RNAse /DNAse free water (WHITE lid)
- 50 x q16 reaction tubes

Reagents and equipment to be supplied by the user.

- q16 instrument
- Easy DNA/RNA Extraction Kit

This kit is designed to work well with all processes that yield high quality DNA, but the Easy extraction method is recommended for ease of use.

- Lab-In-A-Box

The Lab-In-A-Box contains all the pipettes, tips, and racks that you will need to use an Easy kit.

Step-by-step guide

1.Creating our reaction mix

Using the blue pipette to transfer 500µl of the oasis master mix resuspension buffer into the tube of lyophilized oasis master mix and mixing well by inversion. Then transferring all of that master mix into the brown tube labelled*S.negevensis* primers/probe. Capping and shaking tube to mix. A thorough shake was essential to ensure that all components were resuspended. Failure to mix well could produce poor kit performance. Leaving to stand for 5 minutes. Our reaction mix was ready to use. We Stored the reaction mix in the freezer.

2. Internal extraction control

Using the blue pipette to transfer 1000µl (2 x 500µl) of water into the Internal Extraction Control DNA tube. Capping and shaking tube to mix. Our kit contains Internal Extraction Control DNA. This was added to our biological sample at the beginning of the DNA extraction process. It was extracted along with the DNA from our target of interest. The q16 detected the presence of this Internal Extraction Control DNA at the same time as our target. This was the ideal way to show that our DNA extraction process had been successful.

3. Set up a test.

We use the red pipette to combine 10µl of our *S. negevensis* reaction mix with 10µl of our DNA sample in the reaction tubes provided. Always change pipette tips between samples.

4.Negative control

For each test we will require a negative control. Instead of DNA, water is used. This sample should prove negative thus proving that all our positive samples really are positive. Because some kit targets are common in the environment, we may occasionally see a “late” signal in the negative control. The q16 software will take this into account accordingly.

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5.Positive control

Using the blue pipette to transfer 1000µl (2 x 500µl) of water into the positive control template tube. Capping and shaking tube to mix. Each time we run a test we require a positive control. This is a small portion of DNA from our target of interest.

6.Running the test

We Place the tubes into the correct positions in the q16 as defined by the software and start run.

Specificity

The Quantification Kit for *Simkanianegevensis*has been designed for the specific and exclusive in vitro quantification of *Simkanianegevensis*. The 16S ribosomal gene, is the ideal target to achieve a broad-based detection profile for all strains within this species. The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

The Kit for *Simkanianegevensis* (S.negevensis) genomes is designed for the in vitro quantification of *S.negevensis*genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the *S.negevensis* genome. The primers and probe sequences in this kit have 100% homology with a broad range of *S. negevensis* sequences based on a comprehensive bioinformatics analysis.

Ethical considerations:

The study was approved by the Zagazig University institutional review board (IRB), official permission from study setting department and an informed written consent was obtained from all patients before they joined the study.

Statistical analysis: Data collected throughout history, basic clinical examination, laboratory investigations and outcome measures coded, entered, and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) **(Statistical Package for the Social Sciences)** software for analysis.According to the type of data qualitative represent as number and percentage, quantitative continues group represent by mean ± SD, the following tests were used to test differences for significance; difference and association of qualitative variable by Chi square test (X^2). Differences between quantitative independent groups by t test.

3- Sensitivity specificity predictive value

		Condition (as determined by "Gold standard")		
		Positive	***Negative***	
Test outcome	***Positive***	**True Positive**	**False Positive** (Type I error)	→ **Positive predictive value** = $\frac{\Sigma \text{ True Positive}}{\Sigma \text{ Test outcome Positive}}$
	Negative	**False Negative** (Type II error)	**True Negative**	→ Negative predictive value = $\frac{\Sigma \text{ True Negative}}{\Sigma \text{ Test outcome Negative}}$
		↓ Sensitivity = $\frac{\Sigma \text{ True Positive}}{\Sigma \text{ Condition Positive}}$	↓ Specificity = $\frac{\Sigma \text{ True Negative}}{\Sigma \text{ Condition Negative}}$	

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ROC curve

A receiver operating characteristic (ROC), or simply ROC curve, is a graphical plot which illustrates the performance of a binary classifier system as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the positives (TPR = true positive rate) vs. the fraction of false positives out of the negatives (FPR = false positive rate), at various threshold settings. TPR is also known as sensitivity (also called recall in some fields), and FPR is one minus the specificity or true negative rate.

ROC analysis provides tools to select possibly optimal models and to discard suboptimal ones independently from (and prior to specifying) the cost context or the class distribution. ROC analysis is related in a direct and natural way to cost/benefit analysis of diagnostic decision making. The ROC curve was first developed by electrical engineers and radar engineers during World War II for detecting enemy objects in battlefields and was soon introduced to psychology to account for perceptual detection of stimuli. ROC analysis since then has been used in medicine, radiology, biometrics, and other areas for many decades and is increasingly used in machine learning and data mining research.

- Sensitivity = (true +ve)/ [(true +ve) + (false –ve)].
- Specificity = (true –ve) / [(true –ve) + (false +ve)].
- PPV = (true +ve) / [(true +ve) + (false +ve)].
- NPV = (true –ve)/ [(true –ve) + (false –ve)].
- Accuracy = (TP+TN)/[TP+FP+TN+FN]
- The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following:

P value was set at <0.05 for significant results &<0.001 for high significant result.

Results:

Age was distributed as **46.71±10.61** with minimum 23 years and maximum 67 years, regarding sex distribution most cases were males with 88.5% and females were 11.5% **(Table 1)**.

48.7% had DM, 24.4% had HTN, 9.0% had chronic respiratory diseases. According to medical history distribution among hemodialysis patients 7 of them were treated from chronic respiratory diseases (COPD and asthma) **(Table 2)**.

Eight water samples were collected from hemodialysis water from March to November 2020, two samples of them were positive for *Simkania*.During nine months of the study time, the water samples distribution was as follow:

- The 1st ,3rd and fourth months; the water samples for *Simkania* were negative.
- The 5th,6th months; the water samples for *Simkania* turned positive.
- The last 3 months 7th,8th and 9th, the water samples taken turned negative.
- *Simkania* positive founded in 25% of studied samples.**(Table 3)**.

This table shows no significant difference regarding age, sex, history of DM and HTN between the patients undergoing hemodialysis with negative water sample and positive water sample for Simkania**(Table 4).**

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There was no significant difference between groups regarding the lab parameters, CBC, kidney function tests and liver function tests(**Table 5**).

- When the water samples were negative for *S.Negevensis*, 39 patients were examined and followed up, including the seven patients with a history of respiratory diseases .Only 2 of 39 patients developed respiratory symptoms and pulmonary infiltrates in radiology.
- When the water samples were positive for *S. Negevensis*,78 patients were examined and followed up 15 of 78 patients developed respiratory symptoms and pulmonary infiltrates in radiology.
- When the patients were undergoing hemodialysis with positive *Simkania* in the water samples, we had a significant association with the respiratory symptoms compared to hemodialysis with negative water samples for *Simkania*(**Table 6**).

Serum Ferritin and IgG levels are significantly higher in patients during hemodialysis when the water samples were positive for Simkania(**Table 7**).

Significant area under curve with significant cutoff >63.5 for ferritin and >6.1 for IgG with sensitivity 62.0% & 73.3% and specificity 65.5% and 80.0% respectively(**Table 8 & figure 1**).

Table1: Age and sex distribution among studied group

		Age (years)	
Mean± SD		**46.71±10.61**	
Median (Range)		**50.0 (23-67)**	
		N	**%**
Sex	**Male**	**69**	**88.5**
	Female	**9**	**11.5**
	Total	**78**	**100.0**

Table2: Medical history and characters distribution

		N	%
DM	**No**	**40**	**51.3**
	Yes	**38**	**48.7**
HTN	**No**	**59**	**75.6**
	Yes	**19**	**24.4**
Respiratory diseases (asthma, COPD)	**No**	**71**	**91.0**
	Yes	**7**	**9.0**

Table3:*SimkaniaNegevensis* in water samples

		N	%
Simkania in water sample	**-VE**	**6**	**75**
	+VE	**2**	**25**
	Total	**8**	**100.0**

Table4: Demographic data in patients undergoing hemodialysis with negative water for Simkania compared to hemodialysis with positive water for Simkania.

HD patient's data			**Simkania in water sample test**		**t/ X^2**	**P**
			-VE	**+VE**		
Age (years)			**47.1±11.61**	**45.6±7.02**	**0.544**	**0.588**
Sex	**Male**	**N**	**31**	**69**		
		%	**79.5**	**88.5 %**		

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	Female	N	8	9	0.062	0.803
		%	20.5%	11.5 %		
DM	No	N	20	40		
		%	51.2%	51.3%		
	Yes	N	19	38	0.42	0.512
		%	48.7%	48%		
HTN	No	N	29	59		
		%	74.3%	75.6%		
	Yes	N	10	19	3.01	0.083
		%	25.6%	24.4%		

Table5: Blood tests in patients undergoing hemodialysis with negative water samples for Simkania compared to hemodialysis with positive water for Simkania

Blood tests	Water sample test for Simkania		t/ X^2	P
	-VE (n=39 patients)	+VE (n=78 patients)		
HB(g/dL)	9.88±1.2	10.3±1.18	1.339	0.185
Urea(mg/dL)	151.84±24.81	156.25±15.33	1.728	0.089
Cr(mg/dL)	7.71±1.76	8.33±1.98	1.301	0.197
Total Ca(mg/dL)	8.57±0.30	8.62±0.38	0.600	0.550
po4(mg/dL)	10.01±3.63	12.1±4.25	1.337	0.185
ALT (units/L)	22.36±5.73	19.3±6.38	1.830	0.071
AST (units/L)	18.56±4.58	16.65±5.25	1.412	0.162
MCV (FL)	75.63±5.87	77.10±3.27	1.786	0.087
Total bilirubin(mg/dL)	0.66±0.23	0.73±0.15	1.338	0.185
Albumin(g/L)	3.92±0.37	3.75±0.45	1.669	0.099
Uric acid(mg/dL)	6.20±0.83	6.16±1.05	0.209	0.835

Table6: Clinical data in patients undergoing hemodialysis with negative water samples compared to hemodialysis with positive water for *Simkania*

Clinical data			Water sample test for *Simkania*		t/ X^2	P
			-VE (n=39patients)	+VE (n=78patients)		
*Respiratory symptoms (fever, cough and shortening of breath) and chronic diseases	No	N	37	63	4.0	0.045*
		%	94.8%	80.7%		
	Yes	N	2	15		
		%	5.12 %	19.2%		

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exacerbation						
GIT (vomiting, diarrhea)	**No**	**N**	**26**	**70**	**3.06**	**0.08**
		%	**66.6%**	**89.7%**		
	Yes	**N**	**13**	**8**		
		%	**33.3%**	**10.2%**		

Table7: comparison between patients during hemodialysis with *Simkania* positive and negative water samples regarding serum Ferritin and IgG levels

	Water sample test for Simkania		**t/ Mann Whitney**	**P**
	-VE	**+VE**		
Ferritin (ng/mL)	**57.27±17.5**	**67.8±21.85**	**3.020**	**0.002***
IgG (mg/mL)	**4.06±1.81**	**7.52±2.95**	**4.179**	**0.00****

Figure (1) ROC Curve for detection of Ferritin and IgG cutoff for positive *Simkania* water sample

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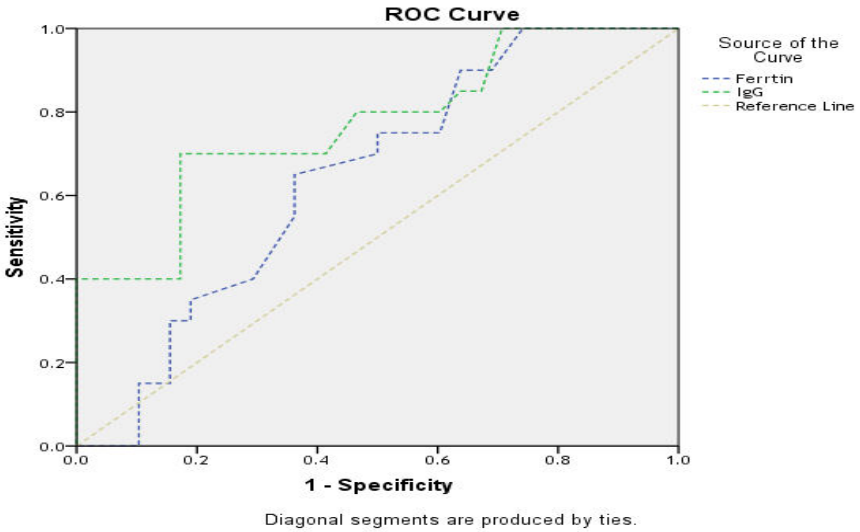

Table8: AUC, cutoff, and validity

Test Result Variable(s)	Area	Cutoff	P	95% Confidence Interval		Sensitivity	Specificity
				Lower Bound	Upper Bound		
Ferritin (ng/mL)	**0.677**	**>63.5**	**0.045***	**0.770**	**0.965**	**62.0%**	**65.5%**
IgG (mg/mL)	**0.770**	**>6.1**	**0.00****	**0.645**	**0.895**	**73.3%**	**80.0%**

Discussion

In our study we investigate the occurrence of *S. Negevensis* infection in the hemodialysis patient.

According to our study, Total of 8 water samples were collected from HD tap water in different occasions after the specific treatment of disinfection from March to November 2020. Two positive water samples were found from total of eight water samples. This is probably due to lack of proper periodic disinfection procedures in water treatment system. But the study of ***angletti et al.*** [5]collected the water samples half of them from tap sited before specific treatment of disinfection and a half of them after specific treatment of disinfection.

During the study time from March to November, the water samples distribution was as follow:

- The 1st ,3rd and fourth months; the water samples for *Simkania* were negative.
- The 5th,6th months; the water samples for *Simkania* turned positive.
- The last 3 months 7th,8th and 9th, the water samples taken turned negative.

In the first 4 months of our study when the water samples were negative for *Simkania*, 39 patients were selected randomly, IgG antibodies were measured in their serum and the routine laboratory investigations were established for these patients. When the negative water samples turned positive, we measured the serum IgG for all the patients included in our study (78 patients), and the routine laboratory investigations were also established for all patients.

Regarding age and sex distribution among studied group. Mean Age was 50 years distributed as (46.71±10.61 SD) with minimum 23 years and maximum 67 years, regarding sex distribution, most cases were males with 88.5% and females were 11.5

There was no difference in the significance of age and gender between the patients undergoing hemodialysis with negative water samples for Simkania compared to hemodialysis with positive

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water for S.Negevensis.In contrast to the study of ***AL-Younes and Paldanius***[(6)] which was performed in Jordan ,the study demonstrated a possible relationship between gender and the prevalence of the bacterial infection of Simkania .

Regarding HTN and DM distribution among studied groups, there was no significant difference between the patients undergoing hemodialysis with negative water samples for *Simkania* compared to hemodialysis with positive water for *S.Negevensis.*

Hemoglobin level is the most specific parameter used to establish the presence and severity of anemia in hemodialysis patients [(7)]**.** Hematocrit level is not assessed because it is a relatively unstable parameter and lack standardization [(8)]**.**

There was no significance in our study regarding hemoglobin between patients who were undergoing hemodialysis with positive and negative water samples for Simkania,this agrees with results of **Angletti et al.,** [(5)]study that detected no statistically difference according to hemoglobin and routine investigations in patients and found no relation with the prevalence of the infection of Simkania.

The water that is used in hemodialysis may be involved as a possible source of *S. Negevensis* infection. Mostly important, the forced continuous contact of the HD subjects with *S. Negevensis* could represent one of the several factors, not fully known, implicated in the uremic inflammation [(5)]

IgG levels were significantly higher in patients during hemodialysis when the water samples were positive for Simkania Significant area under curve with significant cutoff >6.1 for IgG with sensitivity 73.3% and specificity 80.0%. This agree with the result of **Donati *et al.*** [(4)]study thatdemonstrated remarkable differences in serum IgG rates against *S. negevensis* and that infection with *Simkania*.

This agrees with the study of **Anglettiet *al.*** [(5)]that demonstrateda high prevalence of IgG antibodies to *S. negevensis* in patients undergoing chronic hemodialysis.

The prevalence and pathogenic potential role of *S. Negevensis* in renal transplant (RT) recipients and in HD patients is still unknown. IgA and IgG antibodies are antibodies produced by the immune system as a response to the infection with *S. Negevensis*. This investigation was prompted by previous *S. Negevensis* detection in water sources and by its relative resistance to chlorination procedures used for routine treatment of drinking water supplies [(7).]

Conclusion:

Our study detected the occurrence of *S.negevensis* in hemodialysis patients. When the patients were undergoing hemodialysis with positive water for *Simkania*, We detected significant rise of IgG antibodies against S. negevensis and significant area under curve with cutoff point >6.1 for IgG with sensitivity of 73.3% and specificity of 80.0%.

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