ORIGINAL ARTICLE

Study on inactivation kinetics of hepatitis A virus and enteroviruses with peracetic acid and chlorine. New ICC/PCR method to assess disinfection effectiveness

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Key words

Disinfection • Peracetic acid • HAV • Enteroviruses • ICC/PCR

Summary

The virucidal activity of chlorine-compounds was studied using hepatitis A virus (HAV) and Poliovirus 2 and comparing the disinfectant efficiency of peracetic acid.
HAV presented a higher resistance to HClO than Poliovirus did. With ClO_2 the inactivation times of HAV were markedly shorter. A comparison between these data and those resulting from the kinetics with peracetic acid (PA) showed that PA is less effective than chlorine.
As a preliminary to future research, the PCR-test integrated with cell-cultures was experimentally introduced for a quick evaluation of the HAV-infectiveness, with the aim of possible application in the field of disinfection and of viruses-isolation from environmental and food samples.

In the food industry, the most widely used disinfectants are in the group of oxidants, including chlorine and its compounds and peracetic acid, which has recently been increasingly used.
The effectiveness of a disinfectant depends on its range of action as well as on the different types of resistance that pathogens have versus disinfectants [1, 2].
Hepatitis A virus (HAV) appears to be the most resistant virus both versus disinfectants and versus environmental conditions. Recent studies have shown that it can continue its infecting effect on non porous surfaces for several days, and this ability is in part due to the fact that – like all viruses lacking an envelope – it aggregates with organic material which offers its protection against the inactivation carried out by disinfectants. Disinfectants with oxidizing effects act on viruses on a level with the lipid membrane or the capsid and/or nucleic acid.
Peracetic acid, for instance, appears to be involved in the destruction of disulphide bonds occurring in the capsid or structural structures of viruses [3, 4].
Studies on the inactivation of HAV have shown that chlorine can damage viral RNA, in particular the 5'NTR region which has been shown to be linked to the infectivity of the virus: in fact, the total inactivation of viral infectivity does not permit polymerase chain reaction (PCR) identification of this region, which is different to other RNA sequences which can be identified under the same operating conditions [5].
Chlorine dioxide appears to inactivate viruses by preventing their replication by damaging the genome, and in the case of the hepatitis A virus by actually damaging the 5'NTR region, as well as by reacting with the proteins in the viral capsid, thereby inhibiting the viral attack on the target cell and its subsequent penetration [6, 7].
Disinfectants may be hindered in their activity by the viruses themselves which implement greater or lesser resistance, as well as by environmental factors such as pH values and the presence of organic substances.
It is mainly sodium hypochlorite which is greatly affected by the presence of organic substances in the matrix which is to be disinfected; in fact, in the presence of precursors it produces as sub-products trihalomethanes, toxic halogenated compounds deriving from chloroform [8, 9].
Several studies have also reported that disinfection of waters by using peracetic acid is affected by the presence of organic substances which sequestrate the disinfectant thereby requiring the use of excess disinfectant.
Chlorine dioxide does not give rise to THM in the presence of organic substances since it is little affected by the latter and is able to react in a wide range of pH values; as a result, it is often preferred to hypochlorite in disinfection, especially for waters for human consumption.
The formation of chlorites should not be underestimated; these are reaction products which can be found in waters that have been disinfected with chlorine dioxide.
The disinfecting effectiveness is tested with experimental tests whose objective is to assess the most suitable combinations of concentration-contact time of the disinfectant required to ensure inactivation of pathogen microorganisms.
In this regard, viruses become indicators of the successful effect of the disinfectant because they are most

resistant to disinfectants and therefore their inactivation confirms that the most sensitive pathogen microorganisms have been inactivated.
The presence or absence of the virus after disinfection is searched for by semination of the virus-disinfectant inoculation in cell cultures, a traditional method for viral identification, or with the more modern methods of molecular biology such as PCR, which amplifies and identifies a target sequence of the genome of the virus being searched for.
Cell cultures and PCR are methods for viral identification which have several limits when used alone.
It is owing to the limits both methods have when used alone that several studies report on the effectiveness of the integration of these two methods of analysis; thus, one speaks of ICC-PCR integration (integration cell-culture-PCR) [10].
This method of viral identification requires semination of the sample one starts off with, often characterized by low viral loads which cannot be identified by PCR alone in cell cultures.
At intervals an aliquot of the culture medium is taken and submitted to PCR: if there is a negative result at T0 time and a positive one at T1 time, this means that at T1 time a multiplication of the virus in culture must have taken place, consequently the virus has not been inactivated as it is multiplying in the cells.

Aims

The aims of this study can be summed up as follows:
1) assessment of the resistance of certain viruses to the effect of hypochlorous acid and chlorine dioxide by studying the inactivation kinetics performed with different concentrations of the disinfectant and with neutral pH;
2) statistical analysis for assessment of the effectiveness of the disinfectants tested for several viruses and under different pH conditions; the analysis will include peracetic acid whose inactivation tests were carried out previously [11];
3) identifying and implementing new fast methods for identification of viruses. The traditional PCR method was integrated with the use of cell cultures to assess the sensitivity of the molecular system and to define a new method to test the effectiveness of the disinfectants involved.

Materials and methods

Kinetics of viral inactivation with hypochlorous acid and chlorine dioxide

Performing the experimental tests

We tested HAV and Poliovirus 2 for hypochlorous acid, and HAV for chlorine dioxide.
Concentration equal to 0.4-0.8-1-1.4-1.6-mg/l of hypochlorous acid and chlorine dioxide, at a temperature of +20 °C and neutral pH came into contact with viral suspensions for different times (0-30”-1’-2’-3’-4’-5’-15’-30’-45’-60’).
Once the reaction was blocked with sodium thiosulphate, the various samples were seminated in selective cell cultures to breed the viruses (FrHK4 monkey foetal kidney cells for HAV and RC-37 monkey kidney cells for Poliovirus 2).
Titres were expressed in UFP for HAV, and in TCID50 for Polio2.

PCR Test

Sensitivity tests with PCR on HAV
Performing the experimental tests: we calculated the minimum detectable concentration of HAV with PCR only.
Scalar dilutions with base 10 were prepared for HAV and submitted to semi nested – PCR after extraction of RNA and RT.

Specific primers for HAV
PCR: AV1:5’-GTCTCAGGTACTTTCTTTG-3’
AV2:5’-GTTTTGCTCCTCTTTATCATGCTATG-3’
Semi nested: AV2:5’-GTTTTGCTCCTCTTTATCATGCTATG-3’
AV3:5’-TCCTCAATTGTTGTGATAGC-3’

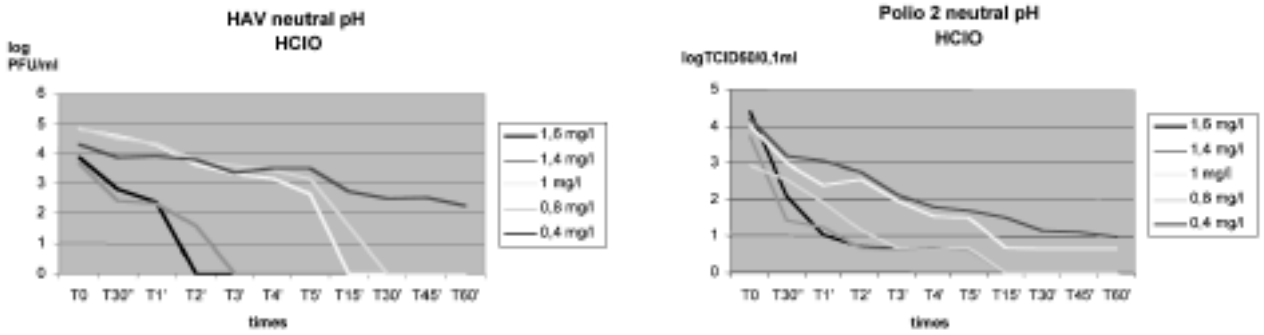
ICC/PCR Tests
Performing the experimental tests: the minimum concentration of HAV detectable with PCR was calculated from the previous tests.
In these tests we used the HAV dilution corresponding to a viral dilution of 1 log lower than the minimum detectable concentration for the previously observed PCR. 0.5 ml of this dilution was inoculated in selective cell cultures for l’HAV.
After different contact times, 0, 6, 24, 48, 72, 96, 120 and 144 hours, samples of the supernatant and of the cell lysate (obtained be freezing and de-freezing infected cells) were taken, and both were then seminated in cell cultures and submitted to RT – nested PCR (the same *primers* as seen before).
Results obtained with PCR were then compared with those obtained after 12 days’ cell culture with visualisation of the cytopathic effect.

Results

Kinetics of viral inactivation with hypochlorous acid and with chlorine dioxide

The graphs show the kinetic patterns for the two disinfectants in the different pH conditions.
In tests carried out on HAV and on Polio 2 at neutral pH with hypochlorous acid, complete inactivation times took from 2 to 30 minutes at concentrations ranging between 0.8 and 1.6 mg/l (Fig. 1).
As regards chlorine dioxide, which has so far been tested only on HAV, inactivation times were markedly

Fig. 1. Inactivation kinetics: HAV and Polio 2 hypochlorous acid.



shorter with concentrations from 0.4 to 0.8 mg/l. At higher concentrations viral activity was absent after the first 30' of contact (Fig. 2).

Statistical analysis

Linear regression

The relations between concentration of the disinfectants tested and inactivation times obtained were analysed by simple regression.

The various tests carried out with peracetic acid showed a good linear relation with R^2 in the range of 0.84 and 0.99, as indicated in the graphs.

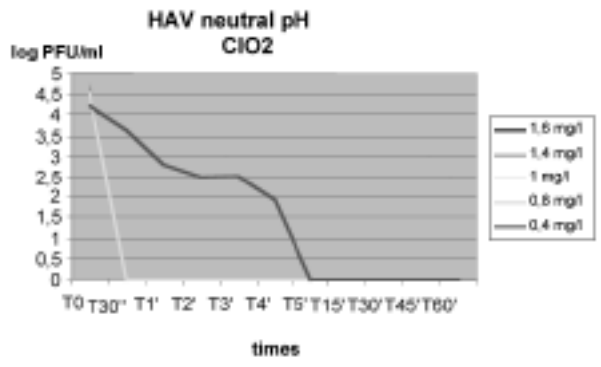
As far as hypochlorous acid is concerned, a good linear pattern appeared only for HAV, while data were more variable for Polio 2.

As for ClO_2, the only data available were for HAV; interpolation was carried out by using only 3 concentrations (0.4-0.6-0.8 mg/l) since at higher concentrations inactivation is > 99.99% within the first 30" and R^2 was equal to 0.81 (Figs. 3, 4, 5, 6, 7, 8).

Comparison of effectiveness of peracetic acid at various pH values (at mean concentration of the test)

By combined variance and regression analysis (Covariance analysis) we proceeded to check the effect of pH on inactivation times for the three viruses (HAV, Polio 2 and Cox B5), using peracetic acid as disinfectant.

Fig. 2. Inactivation kinetics: HAV chlorine dioxide.



At the concentrations of 1-1.4-1.6 mg/L of ClO2 the trend of viral titre is the same as at 0.8 mg/L.

Inactivation times for HAV were not statistically different (P = 0.960) for the three pH values tested (acid, neutral and basic).

When the same analysis was carried out for Poliovirus 2 and for Cox B5, there were significant differences between mean inactivation times in relation to pH; for Cox, F was equal to 13.92 (P = 0.001), and equal to 12.23 (P = 0.001) for Polio 2.

For both Enteroviruses the mean time at basic pH was statistically much higher than at neutral or acid pH; no statistically significant difference was observed between mean times at acid and neutral pH.

Polio 2	acid pH	neutral pH	basic pH
Mean 99% inactivation times (min)	20.11	18.83	53.27
Cox B5	**acid pH**	**neutral pH**	**basic pH**
Mean 99% inactivation times (min)	12.78	11.15	56.22

Comparison between virus resistance to neutral pH with use of peracetic acid and hypochlorous acid (at mean concentration of the test)

The same analysis of covariance was carried out to assess the different resistance of the viruses being tested in relation to peracetic acid and hypochlorous acid at neutral pH.

When using peracetic acid, the analysis showed F = 4.62 (P = 0.029), with mean inactivation time statistically higher for HAV than for Polio 2 and Cox B5.

Mean inactivation times for the two Enteroviruses were statistically different and Polio 2 showed a greater resistance than Cox B5.

Peracetic acid, neutral pH	HAV	Polio 2	Cox B5
Mean 99% inactivation times (min)	76.01	18.83	11.15

When using hypochlorous acid at neutral pH, inactivation times for HAV and for Polio 2 were comparable since the covariance analysis did not show any statistically significant differences (P = 0.179) in viral inactivation times.

Fig. 3. Regression lines: HAV peracetic acid.

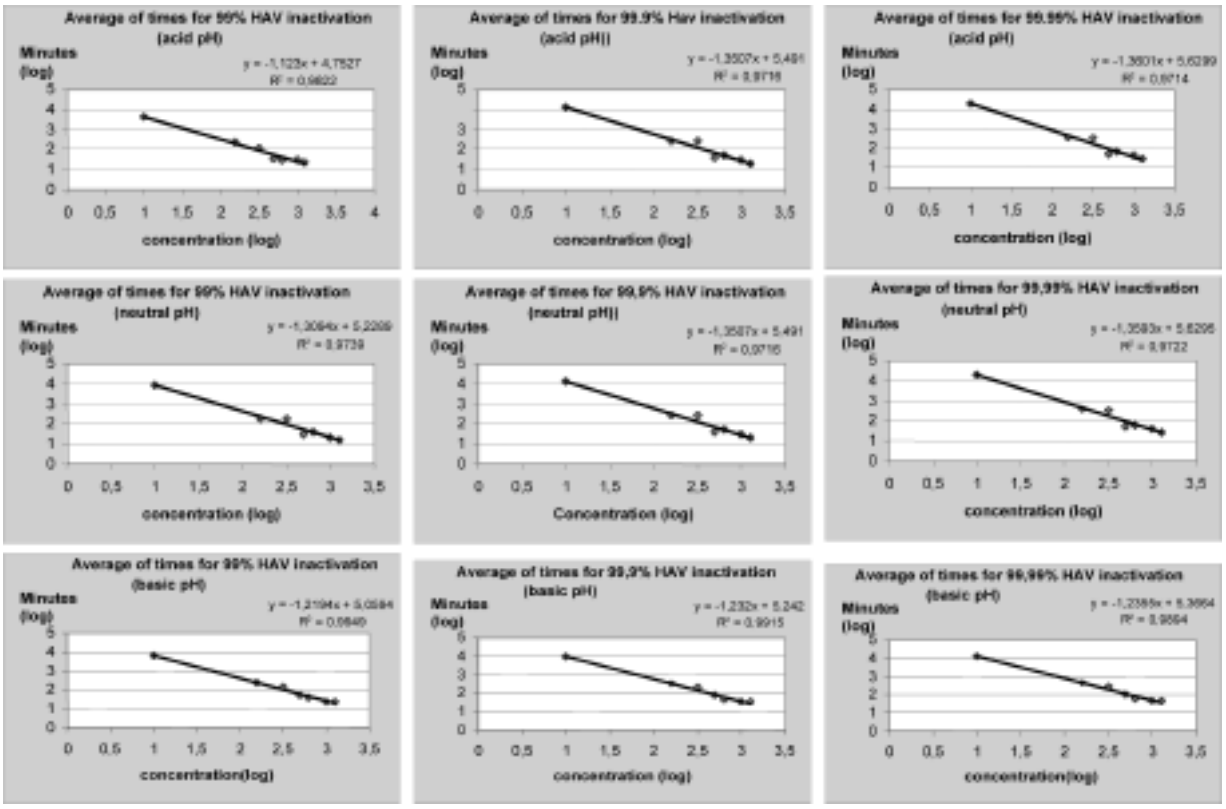
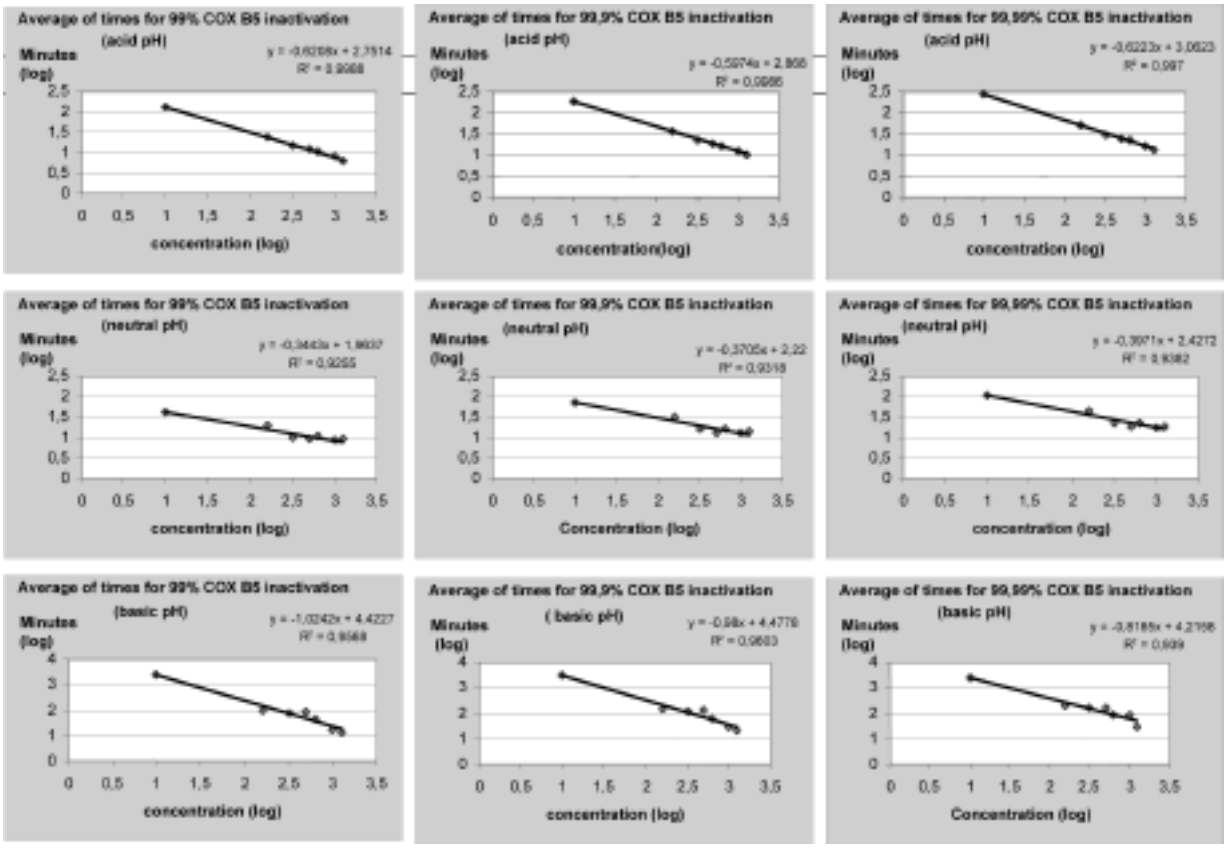


Fig. 4. Regression lines: COX B5 peracetic acid.



Prediction model

By using data from the various tests concerning presence/absence of viral activity under different pH conditions, disinfectant concentrations and contact times, a multiple logistic regression model was made for calculating the probability to obtain inactivation > 99.99% in relation to the parameters considered.

The analysis carried out for single viruses and single disinfectants showed that pH parameters, contact times and concentration were all significantly associated.

We calculated the equations for each virus and relative disinfectant, from which it is possible to calculate the OR (odds ratio), or the probability of inactivation (in fact, the event is determined by the inactivated virus) at fixed punctual values of pH, time, concentration (Tab. I).

PCR Test

PCR sensitivity tests with HAV

The mean HAV titre in the various tests was 10^6 PFU/ml.

The various PCR dilutions testes gave positive values ranging from 10^{-3} to 10^{-4} and were on the whole in agreement with results obtained in cell cultures, which showed cytopathic effect with inoculation of these dilutions (Figs. 9, 10)

ICC/PCR Tests

The first negative dilution at PCR was inoculated in cell-culture in the various tests: at pre-set times an aliquot of the supernatant and an aliquot of the cell lysate were taken as samples. Both aliquots were tested in PCR.

Results showed a positive trend for the test as from 120 hours for the supernatant, and 96 hours for lysate (Figs. 11, 12).

Inoculation in cell culture of the same aliquots of lysate and supernatant showed a notable cytopathic effect in times ranging from 12 to 14 days.

Conclusions

The study of inactivation kinetics with hypochlorous acid on HAV and on Polio 2 showed good oxidizing effects of the disinfectant

From the regression lines with HClO values of 0.4 and 1 mg/l, representing widely used concentrations in disinfection, 99% inactivation times are obtained of 35 and 12 minutes for HAV, and 20 and 7 minutes for Polio 2. These times appear to be quite low for both viruses tested and when comparing them to the covariance analysis they are not significantly different and are totally comparable, showing an equal resistance to the oxidizing effect of the two viruses.

The study of inactivation kinetics with chlorine dioxide, which has only been carried out on HAV so far, has

Fig. 5. Regression lines: Polio 2 peracetic acid.

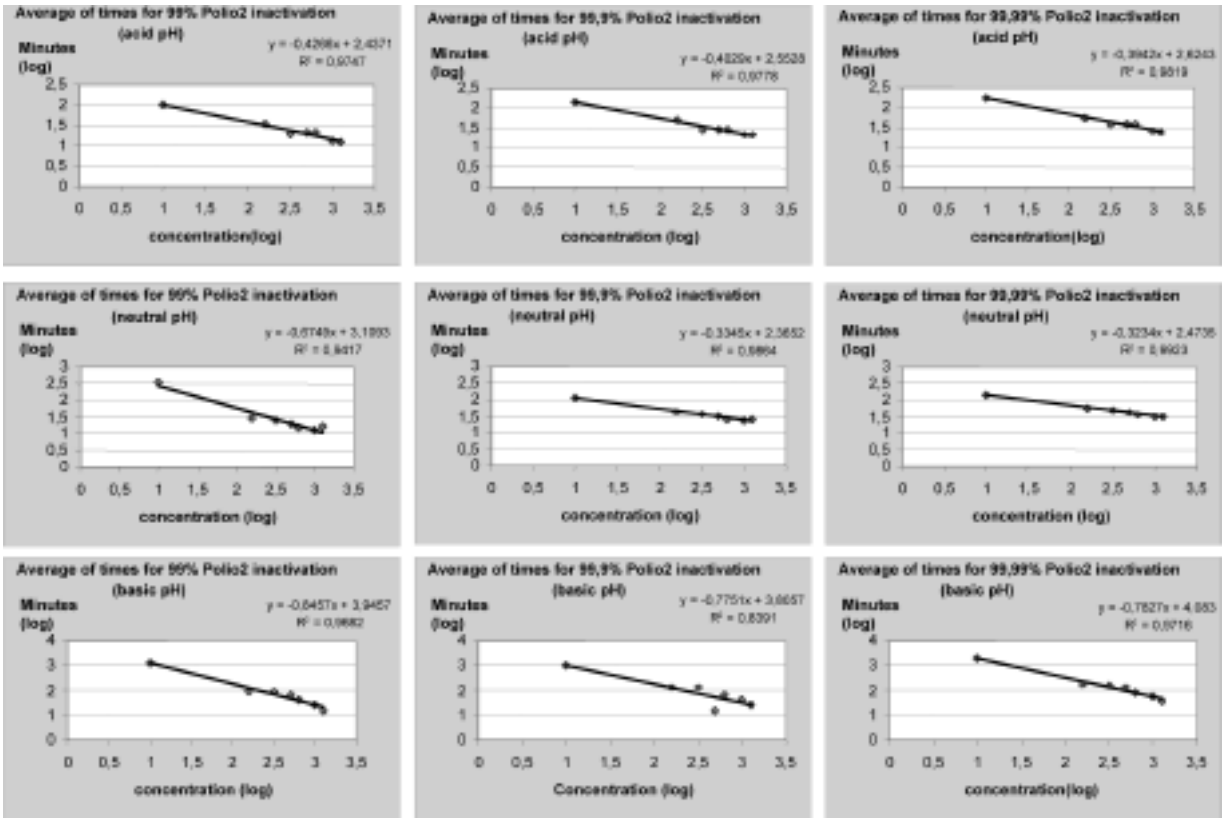


Fig. 6. Regression lines: HAV hypochlorous acid.

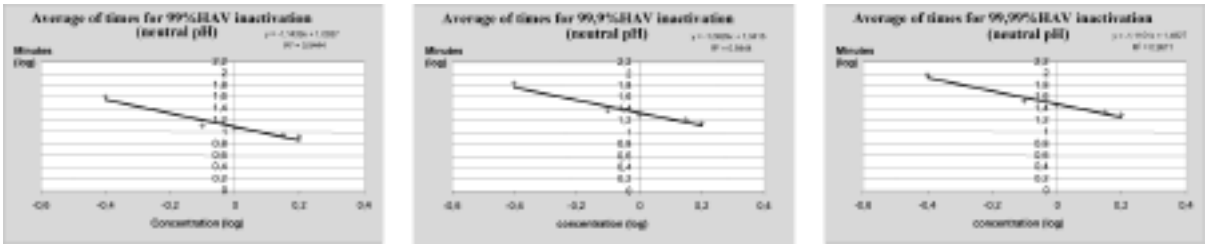


Fig. 7. Regression lines: Polio 2 hypochlorous acid.

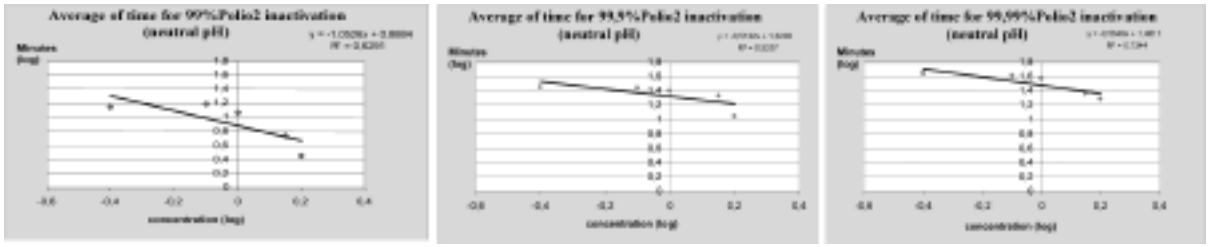
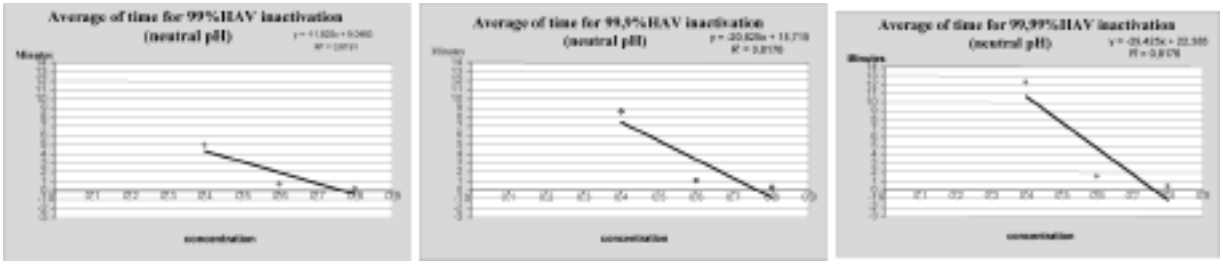


Fig. 8. Regression lines: HAV chlorine dioxide.



shown even lower inactivation times compared to those of hypochlorous acid.

From the regression lines with ClO_2 values of 0.4 and 0.6 mg/l, concentrations which are applicable in practise, very low 99% inactivation times are obtained, equal to 4 and 2 minutes respectively.

If we take into consideration previous data of inactivation kinetics of peracetic acid versus HAV, Polio 2 and Cox B5, it is possible to note a slower effect of the disinfectant as compared to the one of HClO and ClO_2. At 480 and 640 mg/l of peracetic acid at neutral pH, 99% inactivation times were equal to 52 and 36 minutes respectively for HAV, 11 and 10 minutes for Cox B5, and 19 and 16 minutes for Polio 2.

In the case of HAV, which is the most resistant virus, times required to obtain a 99% inactivation with peracetic acid are higher is compared to those of hypochlorous acid and chlorine dioxide, taking into consideration concentrations which can be used in practise for these disinfectants.

In the case of the covariance analysis, by comparing, at the mean concentration of the tests, the resistance of HAV and of Enteroviruses at neutral pH, with the use of peracetic acid, HAV was even more resistant and of the two Enteroviruses Polio 2 has shown a greater resistance than Cox B5. With the same analysis the pH effect was examined on inactivation times of the three viruses; it does not appear to be significant for HAV, which confirms that it is the most resistant and therefore is not significantly affected by the greater oxidizing effect of peracetic acid at acid pH; the two Enteroviruses require longer contact times only with basic pH, while the effect of the disinfectant appears to be similar at acid and neutral pH.

As a preliminary study, we tried to assess the possibility of applying the biomolecular PCR test to check the effectiveness of the disinfectants, overcoming the PCR incapability to distinguish between inactivated and non-inactivated viruses, i.e. between non viable and viable viruses.

At present, in this year's work we have only applied the aspect of cell culture integration/PCR to amplify a sample made artificially negative to PCR and compare the times taken to become positive to PCR for that sample, and the times taken for the cytopathic effect to appear when inoculating the same sample in cell culture.

Results obtained for HAV have shown ICC/PCR times

Tab. I. Prediction models with logistic regression.

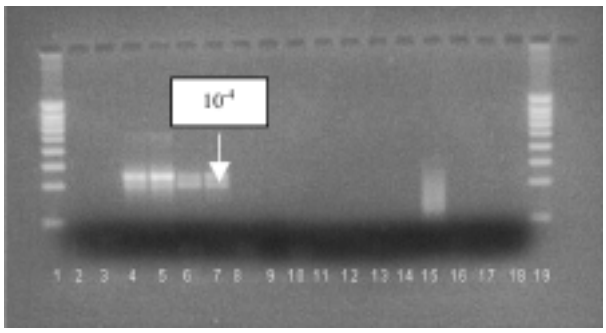
HAV: peracetic acid			
Parameters	B	OR	P
PH	-1.016	0.362 (0.138-0.949)	0.039
Times	0.261	1.299 (1.117-1.510)	0.001
Concentrations PA	0.011	1.011 (1.004-1.017)	0.001
Y = -12.717-1.016 (pH) + 0.261 (time) + 0.011 (conc) *			
Cox: peracetic acid			
Parameters	B	OR	P
PH	-1.869	0.154 (0.061-0.387)	0.001
Times	0.164	1.178 (1.097-1.265)	0.001
Concentrations PA	0.007	1.007 (1.003-1.010)	0.001
Y = 4.124- 1.869 (pH) + 0.164 (time) + 0.007 (conc) *			
Polio 2: peracetic acid			
Parameters	B	OR	P
PH	-1.592	0.203 (0.088-0.469)	0.001
Times	0.134	1.143 (1.076-1.215)	0.001
Concentrations PA	0.008	1.008 (1.004-1.011)	0.001
Y = 1.345-1.592 (pH) + 0.134 (time) + 0.008 (conc) *			
HAV: hypochlorous acid			
Parameters	B	OR	P
Times	0.167	1.182 (1.058-1.320)	0.003
Concentrations HClO	7.878	2638.325 (16.730-416074.97)	0.002
Y = -11.902 + 0.167 (time) + 7.878 (conc) *			
Polio 2: hypochlorous acid			
Parameters	B	OR	P
Times	0.093	1.097 (1.040-1.157)	0.001
Concentrations HClO	3.327	27.863 (1.611-482.008)	0.022
Y = -7.057 + 0.093 (time) + 3.327 (conc) *			

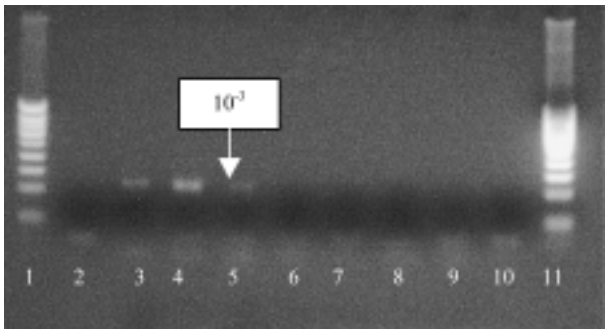
* = Y represents the *log* of the *odds ratio*, i.e. the probability of the inactivation event.Times (min) and concentrations (mg/l) in log

confirming infected viruses 96-144 hours lower than those obtained with the cell cultures alone, in which from 12 to 15 days were needed for the cytopathic effect to appear.

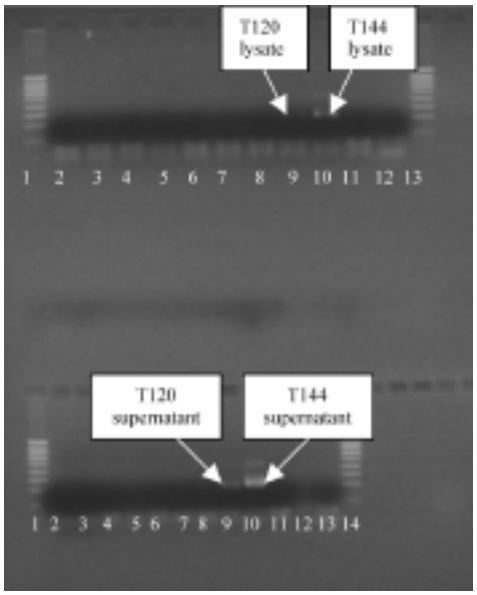
Our work will continue by using samples of inactivated viruses with suitable concentrations and contact times of the disinfectant to assess with the integrated method ICC/PCR, in lower times, if the virus has actually been inactivated, thereby confirming the effectiveness of the disinfection performed.

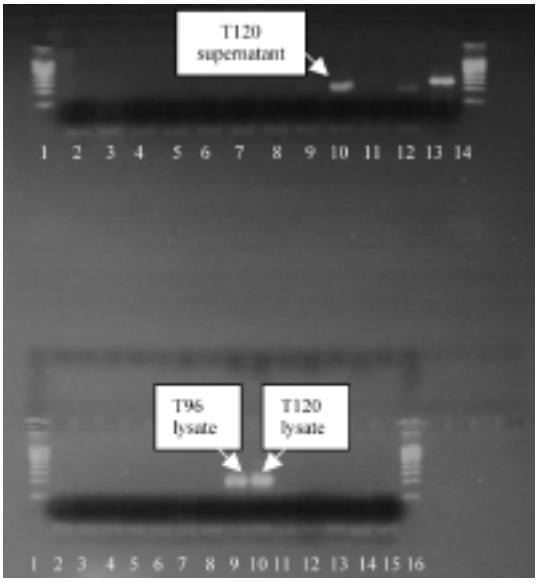
Figs. 9, 10. Sensitivity of PCR test.





Figs. 11, 12. Time of positiveness of PCR starting from negative diluition inoculum.





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